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EXPRESSION CHANGES OF THE ETS FAMILY OF TRANSCRIPTION FACTORS ASSOCIATED WITH CHROMOSOMAL REARRANGEMENTS IN BREAST CANCER

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ABBREVIATIONS

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The following abbreviations, listed in alphabetical order, were used throughout the text:

≥	more or equal to
<	less than
>	more than
µL	microliter
18S rRNA	RNA, 18S ribosomal
3T3-L1	mice (*Mus musculus*) embryonic fibroblastic cell line
ABC reagent	avidin-biotin complex reagent
ADH	atypical ductal hyperplasia
AEG	Crisp 1 alias
AKT	v-akt murine thymoma viral oncogene homolog 1
ALK	anaplastic lymphoma receptor tyrosine kinase
APH	atypical apocrine hyperplasia
BAC	bacteria artificial chromosome
bp	base pair
BSA	bovine serum albumin
CCH	columnar cell hyperplasia
CCL	columnar cell lesion
CDC-2	cyclin-dependent kinase 1

c-Fos	FBJ murine osteosarcoma viral oncogene homolog
CGH	comparative genomic hybridization
CHiP	chromatin immunoprecipitation
CI	confidence interval
CISH	chromagenic *in situ* hybridization
CK(s)	cytokeratin(s)
cm	centimeter
C-MYB	MYB alias
C-MYC	MYC alias
CREB-1	cAMP responsive element binding protein 1
CREM-1	cAMP responsive element modulator
CRISP1	cysteine-rich secretory protein 1
CRISP2	cysteine-rich secretory protein 2
CRISP3	cysteine-rich secretory protein 3
DAB	3,3'-diaminobenzidine
DAPI	4'-6-diamidino-2-phenylindole dihydrochloride
DBD	DNA binding domain
DCIS	ductal carcinoma *in situ*
DDX20	DEAD (Asp-Glu-Ala-Asp) box polypeptide 20
DEAD box	DEAD (Asp-Glu-Ala-Asp) box polypeptide
DNA	deoxyribonucleic acid

E-cad E-cadherin

EDTA ethyleno dyamino tetracetic acid

EHF ets homologous factor

ELF3 E74-like factor 3 (ets domain transcription factor, epithelial-specific)

ELF5 E74-like factor 5

ELK4 ELK4, ETS-domain protein (SRF accessory protein 1)

ER estrogen receptor

ERBB2 codifying gene of Her2/*neu*

ERG v-ets erythroblastosis virus E26 oncogene homolog (avian)

ERK2 MAPK1 alias

ESE epithelial specific ETS

ETS E26 transformation-specific family

ETS1 v-ets erythroblastosis virus E26 oncogene homolog 1 (avian)

ETS2 v-ets erythroblastosis virus E26 oncogene homolog 2 (avian)

ETV1 ets variant 1

ETV3 ets variant 3

ETV4 ets variant 4

ETV5 ets variant 5

ETV6 ets variant 6

FEA flat epithelial atypia

FISH fluorescent *in situ* hybridization

FLI1	friend leukemia virus integration 1
h	hour(s)
H&E	hematoxylin and eosin
Her2/*neu*	Her2 protein
HPRT1	hypoxanthine phosphoribosyltransferase 1
IDC	invasive ductal carcinoma
IDC-NST	invasive ductal carcinoma not otherwise specified
IgePal	octylphenyl-polyethylene glycol
ILC	invasive lobular carcinoma
ISCN	International System for Chromosome Nomenclature
ITC	isolated tumor cells
kDa	kilodaltons
Ki67	monoclonal antibody specific to the protein MKI67
LN	lobular neoplasia
M	molar
MAPK	mitogen-activated protein kinase 1 (official symbol MAPK1)
MAST	microtubule-associated serine-threonine kinase
MCF-12A	human (*Homo sapiens*) epithelial mammary gland immortalized cell line
mg	milligram
MGA	microglandular adenosis
min	minute(s)

mL	milliliter
mm	millimeter
mM	millimolar
MYB	v-myb myeloblastosis viral oncogene homolog (avian)
MYC	v-myc myelocytomatosis viral oncogene homolog (avian)
NaCl	sodium chloride
NaCSN	sodium isothiocyanate
NFIB	nuclear factor i/b
NF-kB	nuclear factor-kappa B
ng	nanogram
NTRK3	neurotrophic tyrosine kinase, receptor, type 3
NUP214	nucleoporin 214kDa
ºC	degree Celsius
PAK1	p21 protein (Cdc42/Rac)-activated kinase 1
PBS	phosphate buffered saline
PgR	progesterone receptor
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (official symbol PIK3CA)
PLCIS	pleomorphic lobular carcinoma *in situ*
PNT	pointed domain
PTEN	phosphatase and tensin homolog
qRT-PCR	quantitative real time PCR

RAS	v-Ki-RAS2 Kirsten rat sarcoma viral oncogene homolog (official symbol KRAS)
RPS6KB1	ribosomal protein S6 kinase, 70kda, polypeptide 1
s	second(s)
SAR	serine and aspartic acid domain
SDS	sodium dodecyl sulphate
SLC45A3	solute carrier family 45, member 3
SRF	serum response factor
SSC	saline-sodium citrate buffer
TCF	ternary complex factor
TGF-β	transforming growth factor beta 1 (official symbol TGFB1)
TGF-βRII	transforming growth factor beta receptor II (official symbol TGFBR2)
TLDA	Taq®Man Low-Density Arrays
TLDU	terminal lobular ductal unit
TMPRSS2	transmembrane protease, serine 2
TWEEN 20	polyethylene glycol sorbitan monolaurate
VMP1	vacuole membrane protein 1
w/v	weight/volume
WHO	World Health Organization

ABSTRACT

ABSTRACT

Several ETS transcription factors are involved in the pathogenesis of human cancers by different mechanisms. As gene copy number gain/amplification is an alternative mechanism of oncogenic activation and 1q gain is the most common copy number change in breast carcinoma, we investigated how that genomic change impacts in the expression of the three 1q ETS family members *ETV3*, *ELK4* and *ELF3*. We have first evaluated 141 breast carcinomas for genome-wide copy number changes by chromosomal CGH, and showed that 1q21 and 1q32 were the two chromosome bands with most frequent genomic copy number gains. Second, we confirmed by FISH with *locus*-specific BAC clones that cases showing 1q gain/amplification by CGH showed copy number increase of the ETS genes *ETV3* (located in 1q21~23), *ELF3* and *ELK4* (both in 1q32). Third, gene expression levels of the three 1q ETS genes, as well as their potential targets *MYC* and *CRISP3*, were evaluated by quantitative real-time PCR. We here show for the first time that the most common genomic copy number gains in breast cancer, 1q21 and 1q32, are associated with overexpression of the ETS transcription factors *ETV3* and *ELF3* (but not *ELK4*) at these *loci* irrespective of molecular subtypes. Among the three 1q ETS genes, *ELF3* has a relevant role in breast carcinogenesis and is also the most likely target of the 1q copy number increase. The basal-like molecular subtype presented the worst prognosis regarding disease-specific survival, but no additional prognostic value was found for 1q copy number status or *ELF3* expression. Additionally, we show that there is a correlation between the expression of the oncogene *MYC*, irrespectively of copy

number gain at its *locus* in 8q24, and the expression of both the transcriptional repressor *ETV3* and the androgen respondent *ELK4*.

RESUMO

RESUMO

Os factores de transcrição ETS estão envolvidos na carcinogénese via diferentes mecanismos. O ganho/amplificação de número de cópias génicas é um dos mecanismos de activação oncogénica. No cancro da mama, o ganho de 1q é a alteração de número de cópias mais comum. Baseando-nos nestes factos, postulámos qual seria o impacto das alterações genómicas na expressão de três genes membros da família dos ETS (*ELF3*, *ETV3* e *ELK4),* cujos *loci* estão em 1q. Avaliámos por CGH cromossómico as possíveis alterações do número de cópias genómicas em 141 carcinomas da mama e demonstrámos que as bandas cromossómicas 1q21 e 1q32 apresentavam a frequência mais elevada de ganhos de cópias cromossómicas. De seguida, confirmámos por FISH com sondas *locus*-específicas obtidas por clones de BACs, que nos casos que apresentavam ganhos/amplificações de 1q por CGH também se verificava o aumento de número de cópias de *ETV3* (*loci* 1q21~23), *ELF3* e *ELK4* (ambos localizados em 1q32). Avaliámos os níveis de expressão génica por PCR quantitativo em tempo real dos três genes ETS localizados em 1q, bem como potenciais genes alvo, nomeadamente os genes *MYC* e *CRISP3*. Assim, neste trabalho reportámos pela primeira vez que os ganhos de cópias genómicos mais comuns no cancro da mama, 1q21 e 1q32, estão associados com a sobre-expressão dos factores de transcrição *ETV3* e *ELF3* (o mesmo não se verificando para o *ELK4*), independentemente do subtipo molecular. De entre os três genes ETS localizados em 1q, o *ELF3* tem um papel relevante na carcinogénese mamária e é o alvo mais provável do aumento de número de cópias de 1q. O subtipo molecular *basal-like* apresentou o pior prognóstico analisando a sobrevivência específica de

doença, mas não se verificou valor de prognóstico adicional no que concerne ao número de cópias de 1q ou à expressão de *ELF3*. Adicionalmente, demonstrou-se que existe uma correlação entre a expressão do oncogene *MYC*, independentemente do ganho do número de cópias no *locus* 8q24, e a expressão do repressor transcripcional *ETV3* e do androgénio dependente *ELK4*.

INTRODUCTION

INTRODUCTION

Breast cancer is the most common malignancy among western women and the second cause of cancer death (1). About 40 000 women die each year in the United States of metastatic breast cancer and the mortality rate is higher in developing countries, making this disease a public health problem (1, 2). The disease incidence is increasing due to, among other factors, the implementation of screening programs and population ageing (3). The decline of the mortality rate during the last two decades from 30% to 20%, despite the aggressiveness of the conventional therapies and the early detection of patients at initial stages of the disease, is far from the desirable (4). Metastatic breast cancer is still clinically incurable (4). In Portugal, breast cancer is the malignancy with the highest incidence and the second cause of death by cancer among women (5).

1. BREAST DISEASES

1.1. BREAST TISSUE AND ASSOCIATED PATHOLOGIES

To better understand breast pathologies, the contextualization of normal breast tissue anatomy is a requirement. Breasts are composed of specialized epithelium and stroma that may give rise to both benign and malignant lesions (6, 7).

The epithelial component consists of a series of branching ducts which bond the structural and functional units of the breast, i.e., the lobules to the nipple (Figure 1).

The stroma, which comprises almost all breast volume in the nonlactational state, is constituted of variable amounts of adipose and fibrous connective tissues (6, 7). Successive branching of the large ducts ultimately leads to the terminal lobular duct unit (TLDU). Each ductal system often occupies more than one quadrant of the breast and they extensively overlap one another (6, 7).

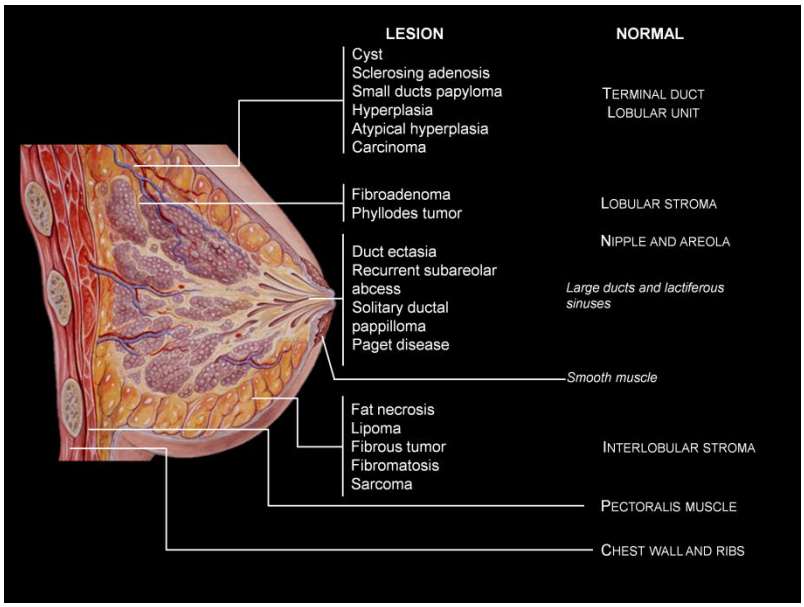

FIGURE 1: Anatomical origin of breast lesions [adapted from (7, 8)].

Throughout women's reproductive life time, during menstrual cycles, pregnancy, breastfeeding and menopause, the breast tissue is exposed to several boosts of hormonal driven cycles, which result in variations of the tissue composition of the organ (4, 5). These proliferation and differentiation cell cycles are DNA error prone and increase the risk of tumor development.

The clinical presentation of breast pathologies more frequently reported by women are mastalgia or mastodynia, nipple discharges and palpable mass or lumpiness (7).

1.2. BREAST CANCER

1.2.1. RISK AND PROTECTIVE FACTORS

The breast is a hormonal dependent tissue and, consequently, among the risk factors that have been associated with breast cancer, gender is the most important, as reflected by male breast cancer representing only 1% of the new breast cancer cases diagnosed (9, 10). Age is also associated with augmented risk of developing breast cancer: the incidence of the disease rises in elderly women and the peak occurs at the age of 70-80 years, being 61 years the average age at diagnosis for Caucasian women and 56 and 46 years for Hispanic and

African-American women, respectively (1, 7, 9). The reproductive maturation and all the events associated with hormonal cycles are also risk factors, namely (7):

- **Age at menarche and menopause**: women that reach menarche younger than 11 years old have 20% increased risk comparing to women in whom it occurs latter than 14 years; late menopause also increases the risk of breast cancer development (11);

- **Age at first live birth and breast feeding**: the first full term pregnancy at ages younger than 20 years reduce breast cancer risk in 20%, comparing to women having the first child after the age of 35 years. Regarding breast feeding, women who breastfeed for longer periods have a decreased risk of developing the disease;

- **Estrogen exposure**: hormonal replacement therapy increases the risk 1.2 to 1.7 fold, and adding progesterone increases the risk even further. Oral contraception is not consensually associated with breast cancer risk reduction, but is associated with diminished risk of ovarian and endometrial cancers.

Another group of risk factors is the personal and the family history of breast pathologies. Breast cancer risk increases with the number of affected first degree relatives and a history of biopsies for atypical hyperplasias, especially when detected, increases the risk of invasive carcinomas. After a primary breast cancer event, the risk of developing a contralateral tumor raises 1% per year (7).

Other risk factors are (7):

- **Race/ethnicity**: Caucasian women present the highest risk of developing breast cancer;

- **Breast density**: anatomically dense breasts have higher risk of developing neoplastic processes due to breast physiology and also to the obstacles to mammographic screening;

- **Radiation exposure**: due to treatments, accidents or labor activities, radiation exposure increases the probability of developing breast cancer;

- **Geographic influence, diet, obesity and exercise**: breast cancer incidence in the United States and in Europe is four times higher than in other countries. Healthy life style habits are not consensually associated with diminished breast cancer risk, but are clearly coupled to less morbidity;

- **Smoking habits**: tobacco is not clearly associated with breast cancer, although it is an obvious risk factor for health status in general.

1.2.2. DIAGNOSIS

The majority of breast cancer patients presents clinical signals and symptomatology, such as lumps, nipple discharges, areola alterations, or even skin deformations. These common findings in symptomatic patients may be accompanied by pain, but an increasing proportion of breast cancers are being diagnosed in asymptomatic phases due to mammographic screening (3).

Traditionally, breast abnormalities should be evaluated by triple assessment, including clinical, imaging (mammographic and ultrasound) and anatomopathological evaluation (by aspirative cytology or biopsy). Based on the preoperative clinical diagnosis estimated by the integration of the histological type/grade of the tumor (Tables 1 and 2), clinical TNM staging (Table 3) and estrogen receptor (ER), progesterone receptor (PgR) and Her2-*neu* status, assessed when primary systemic therapy is planned, the treatment scheme can be designed (3).

TABLE 1: Histological breast cancer classification [adapted from (12)].

HISTOLOGICAL TYPE	ICD-O*
Epithelial tumors	
Invasive ductal carcinoma, not otherwise specified	8500/3
Mixed type carcinoma	
Pleomorphic carcinoma	8022/3
Carcinoma with osteoclastic giant cells	8035/3
Carcinoma with choriocarcinomatous features	
Carcinoma with melanotic features	
Invasive lobular carcinoma	8520/3
Tubular carcinoma	8211/3
Invasive cribiform carcinoma	8201/3
Medullary carcinoma	8510/3
Mucinous carcinoma and other tumors with abundant mucin	
Mucinous carcinoma	8480/3
Cystoadenocarcinoma and columnar cell mucinous carcinoma	8480/3
Signet ring carcinoma	8490/3
Neuroendocrine tumors	
Solid neuroendocrine carcinoma	
Atypical carcinoid tumor	8249/3
Small cell/oat cell carcinoma	8041/3
Large cell neuroendocrine carcinoma	8013/3
Invasive papilary carcinoma	8503/3
Invasive micropapilary carcinoma	8507/3
Apocrine carcinoma	8401/3
Metaplastic carcinoma	8575/3
Pure epithelial metaplastic carcinoma	8575/3
Squamous cell carcinoma	8070/3
Adenocarcinoma with spindle cell metaplasia	8572/3
Adenosqamous carcinoma	8560/3
Mucoepidermoid carcinoma	8430/3
Mixed epithelial/mesenchymal metaplastic carcinoma	8575/3
Lipid-rich carcinoma	8314/3
Secretory carcinoma	8502/3
Oncocytic carcinoma	8290/3
Adenoid cystic carcinoma	8200/3
Acinic cell carcinoma	8550/3
Glycogen-rich cell carcinoma	8315/3
Sebaceous carcinoma	8410/3
Inflammatory carcinoma	8530/3
Lobular neoplasia	
Lobular carcinoma *in situ*	8520/2
Intraductal proliferative lesions	
Usual hyperplasia	
Flat epitheilial atypia	
Atypical ductal hyperplasia	
Ductal carcinoma *in situ*	8500/2
Microinvasive carcinoma	

HISTOLOGICAL TYPE	ICD-O*
Epithelial tumors (cont.)	
Intraductal papillary neoplasms	
Central papilloma	8503/0
Peripheral papilloma	8503/0
Atypical papilloma	
Intraductal papillary carcinoma	8503/2
Intracystic papillary carcinoma	8504/2
Benign epithelial proliferations	
Adenosis including variants	
Sclerosing adenosis	
Apocrine adenosis	
Blunt duct adenosis	
Microglandular adenosis	
Adenomyoepithelial adenosis	
Radial scar/complex sclerosing lesion	
Adenomas	
Tubular adenoma	8211/0
Lactating adenoma	8204/0
Apocrina adenoma	8401/0
Pleomorphic adenoma	8940/0
Ductal adenoma	8503/0
Myoepithelial lesions	
Myoepethiliosis	
Adenomyoepithelial adenosis	
Adenomyoepithelioma	8982/0
Malignant myoepthelioma	8982/3
Mesenchymal tumors	
Haemangioma	9120/0
Angiomatosis	
Haemangiopericytoma	9150/1
Pseudoangiomatous stromal hyperplasia	
Myofibroblastoma	8825/0
Fibromatosis (aggressive)	8821/1
Inflammatory myofibroblastic tumor	8825/1
Lipoma	8850/0
Angiolipoma	8861/0
Granular cell tumor	9580/0
Neurofibroma	9540/0
Schwonnoma	9560/0
Angiosarcoma	9120/3
Liposarcoma	8850/3
Rhabdomyosarcoma	8900/3
Osteosarcoma	9180/3
Leiomyoma	8890/0
Leiomyosarcoma	8890/3

HISTOLOGICAL TYPE	ICD-O*
Fibroepithelial tumors	
Fibroadenoma	9010/0
Phyllodes tumors	9020/1
Benign	9020/0
Borderline	9020/1
Malignant	9020/3
Periductal stromal sarcoma, low grade	9020/3
Mammary hamartoma	
Tumors of the nipple	
Nipple adenoma	8506/0
Syringomatous adenomas	8407/0
Paget disease of the nipple	8540/3
Malignant lymphoma	
Difuse large B-cell lymphoma	9680/3
Burkitt lymphoma	9687/3
Extranodal marginal-zone B cell lymphoma of MALT cell type	9699/3
Follicular lymphoma	9690/3
Metastatic tumors	
Tumors of the male breast	
Gynaecomastia	
Carcinoma	
Invasive	8500/3
In situ	8500/2

Legend: *Morphology code of the International Classification of Diseases for Oncology (ICD-O) and the Systematized Nomenclature of Medicine (12); /0 for benign tumors, /1 for borderline or uncertain behavior, /2 for *in situ* carcinomas and grade 3 intraepithelial neoplasias, and /3 for malignant tumors [extracted from (12)].

TABLE 2: Breast cancer grading: Elston-Ellis system [adapted from (12)].

FEATURE	SCORE
Tubule and gland formation	
Majority of tumors (>75%)	1
Moderate degree (10-75%)	2
Little or none (<10%)	3
Nuclear pleomorphism	
Small, regular uniform cells	1
Moderate increase in size and variability	2
Marked variation	3
Mitotic counts	
Dependent on the microscope field area	1-3

TABLE 3: TNM classification of carcinomas of the breast [adapted from (12)].

TNM CLINICAL CLASSIFICATION	
T-Primary Tumor	
Tx	Primary tumor cannot be accessed
T0	No evidence of primary tumor
Tis	Carcinoma *in situ*
Tis (DCIS)	Ductal carcinoma *in situ*
Tis (LCIS)	Lobular carcinoma *in situ*
Tis (Paget)[1]	Paget disease of the nipple with no tumor
T1	Tumor 2cm or less in greatest dimension
T1mic	Microinvasion 0.1cm or less in greatest dimension[2]
T1a	More than 0.1cm but not more than 0.5cm in greatest dimension
T1b	More than 0.5cm but not more than 1cm in greatest dimension
T1c	More than 1cm but not more than 2cm in greatest dimension
T2	Tumor more than 2cm but not more than 5cm in greatest dimension
T3	Tumor more than 5cm in greatest dimension
T4	Tumor of any size with direct extension to chest wall or skin only as described in T4a to T4d
T4a	Extension to chest wall
T4b	Edema (including *peau d'orange*), or ulceration of the skin of the breast, or satellite skin nodules confined to the same breast
T4c	Both 4a and 4b, above
T4d	Inflammatory carcinoma[3]
N – Regional Lymph Nodes	
NX	Regional lymph nodes cannot be assessed (e.g. previously removed)
N0	No regional lymph node metastasis
N1	Metastasis in movable ipsilateral axillary lymph node(s)
N2	Metastasis in fixed ipsilateral axillary lymph node(s) or in clinically apparent[4] ipsilateral internal mammary lymph node(s) in the absence of clinically evident axillary lymph node metastasis
N2a	Metastasis in axillary lymph node(s) fixed to one another or to other structures
N2b	Metastasis only in clinically apparent[4] internal mammary lymph node(s) and in the absence of clinically evident axillary lymph node metastasis
N3	Metastasis in ipsilateral infraclavicular lymph node(s) with or without axillary lymph node involvement; or in clinically apparent[4] ipsilateral internal mammary lymph node(s) in the presence of clinically evident axillary lymph node metastasis; or metastasis in ipsilateral supraclavicular lymph node(s) with or without axillary or internal mammary lymph node involvement
N3a	Metastasis in infraclavicular lymph node(s)
N3b	Metastasis in internal mammary and axillary lymph nodes
N3c	Metastasis in supraclavicular lymph node(s)
M – Distant Metastasis	
MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

TNM CLINICAL CLASSIFICATION

pTNM Pathological Classification

pT The pathological classification requires the examination of the primary carcinoma with no gross tumor at the margins of resection. A case can be classified pT if there is only microscopic tumor in a margin. The pT categories correspond to the T categories[1]

pN	**Regional Lymph Nodes**
pNX	Regional lymph nodes cannot be assessed (not removed for study or previously removed)
pN0	No regional lymph node metastasis[5]
pN1mi	Micrometastasis (larger than 0.2mm, but none larger than 2mm in greatest dimension
pN1	Metastasis in 1-3 ipsilateral axillary lymph node(s), and/or in internal mammary nodes with microscopic metastasis detected by sentinel lymph node dissection but not clinically apparent[6]
pN1a	Metastasis in 1-3 axillary lymph node(s), including at least one larger than 2mm in greatest dimension
pN1b	Internal mammary lymph nodes with microscopic metastasis detected by sentinel lymph node dissection but not clinically apparent
pN1c	Metastasis in 1-3 axillary lymph node(s) and internal mammary lymph nodes with microscopic metastasis detected by sentinel lymph node dissection but not clinically apparent
pN2	Metastasis in 4-9 ipsilateral axillary lymph nodes, or in clinically apparent[7] ipsilateral internal mammary lymph node(s) in the absence of axillary lymph node metastasis
pN2a	Metastasis in 4-9 axillary lymph nodes, including at least one that is larger than 2mm
pN2b	Metastasis in clinically apparent internal mammary lymph node(s), in the absence of axillary lymph node metastasis
pN3	Metastasis in 10 or more ipsilateral axillary lymph nodes; or in infraclavicular lymph nodes; or in clinically apparent ipsilateral internal mammary lymph nodes in the presence of one or more positive axillary lymph nodes; or in more than 3 axillary lymph nodes with clinically negative, microscopic metastasis in internal mammary lymph nodes; or in ipsilateral supraclavicular lymph nodes
pN3a	Metastasis in 10 or more axillary lymph nodes (at least one larger than 2mm) or metastasis in infraclavicular lymph nodes
pN3b	Metastasis in clinically apparent internal mammary lymph node(s) in the presence of one or more positive axillary lymph node(s); or metastasis in more than 3 axillary lymph nodes and in internal mammary lymph nodes with microscopic metastasis detected by sentinel lymph node dissection but not clinically apparent
pN3c	Metastasis in supraclavicular lymph node(s)
pM – Distant Metastasis	
The pM categories correspond to the M categories	

Legend: [1]Paget disease associated with a tumor is classified according to the size of the tumor. [2]Microinvasion is the extension of cancer cells beyond the basement membrane into the adjacent tissues with no focus more than 0.1cm in greatest dimension. When there are multiple foci of microinvasion, the size of only the largest focus is used to classify the microinvasion (the sum of all individual foci should not be used).

TABLE 3 – Legend (cont): The presence of multiple foci of microinvasion should be noted, as it is with multiple larger invasive carcinomas. [3]Inflammatory carcinoma of the breast is characterized by diffuse, brawny induration of the skin with an erysipeloid edge, usually with no underlying mass. If the skin biopsy is negative and there is no localized measurable primary cancer, the T category is pTX when pathologically staging a clinical inflammatory carcinoma (T4d). Dimpling of the skin, nipple retraction, or other skin changes, except those in T4b and T4d, may occur in T1, T2, or T3 without affecting the classification. [4]Clinically apparent = detected by clinical examination or by imaging studies (excluding lymphoscintigraphy). [5]When classifying pT the tumor size is a measurement of the invasive component. If there is a large *in situ* component (e.g. 4cm) and a small invasive component (e.g. 0.5cm), the tumor is coded pT1a. Cases with only isolated tumor cells (ITC) in regional lymph nodes are classified as pN0. ITC are single tumor cells or small clusters of cells, not more than 0.2mm in greatest dimension, that are usually detected by immunohistochemistry or molecular methods but which may be verified on H&E stains. ITCs do not typically show evidence of metastatic activity (e.g., proliferation or stromal reaction). [6]Not clinically apparent = not detected by clinical examination or by imaging studies (excluding lymphoscintigraphy). [7]Clinically apparent = detected by clinical examination or by imaging studies (excluding lymphoscintigraphy) or grossly visible pathologically [extracted from (12)].

1.2.3. BREAST CANCER CLASSIFICATION

Breast cancer can be classified according to several features, namely tissue histology, grade, stage and phenotypical/genomic markers.

1.2.3.1. BREAST CANCER HISTOLOGICAL CLASSIFICATION

Breast carcinomas can be divided into *in situ* and invasive lesions. The carcinoma *in situ* is a neoplastic proliferation limited to the ducts and lobules by the basement membrane, whereas invasive carcinoma has already penetrated through the basement membrane into the stroma, and the cells have the potential to invade into the vasculature and thereby reach the regional lymph nodes and distant sites (7).

The initial taxonomy of breast lesions reflected the assumption that breast cancers would arise from distinct microanatomical structures of the normal breast, hence the terminology of duct and lobular carcinomas. Latter, Wellings *et al* (13-17), called into question this assumption as they proved that the vast majority of these lesions originate from the TLDUs regardless of histological type. Hence, the terms duct and lobular carcinoma do not entail the histogenesis within the mammary ductal system, rather these entities are defined by their discrete architectural patterns, cytological features and immunohistochemical profiles (18). This was a paradigm shift at the time and culminated in the perception that the origin of the invasive lesions results in several histogenetic models of breast cancer

progression. Additionally molecular data now available suggest that *in situ* lesions are at least as heterogeneous as their invasive counterparts (19). Nevertheless, nowadays this terminology still persists.

Besides the cell of origin, the diversity of clinical behavior and biological features that breast cancer presents highlighted the need to categorize the disease into meaningful clusters, in order to easily plan the therapeutic schemes and improve the prognosis. During the last decades the committed efforts for establishing a working flow chart model for breast cancer taxonomy have been put forward, but they were not as successful as expected. Breast cancers are often composed of areas that harbor distinct morphological patterns (18), which is a drawback to an easy and expeditious taxonomical approach. Several breast cancer histological classifications are currently in use (18), but the latest World Health Organization (WHO) classification provides a valuable operational flow through model for breast cancer taxonomy and recognizes the existence of 18 types of breast cancer (Table 1) (12, 18).

Invasive ductal carcinomas not otherwise specified (IDC-NST) account for the large majority (50-80%) of breast carcinomas. This group comprises adenocarcinomas that fail to exhibit enough characteristics to warrant their classification into one of the special types (18). The last edition of WHO classification of breast cancer recognizes the existence of at least 17 histological special types, namely the lobular and medullar invasive carcinomas (12, 18) (Table 1).

1.2.3.2. BREAST CANCER GRADING

Histological grade in parallel with histological type are the two most important tumor intrinsic features that can be determined by histopathological analysis of a breast cancer specimen. Tumor grade is an assessment of the degree of differentiation (that is, tubule formation and nuclear pleomorphism) and proliferative activity (the mitotic index) of a tumor and indicates the tumor aggressiveness (18). Due to interobserver disagreement, the need of easily classifying systems urged. With the introduction of the Nottingham histological grading system based on Elston-Ellis grading system (Table 2) and the

standardization of criteria through external quality assurance schemes, this pitfall has been avoided. In fact, recent studies have demonstrated a moderate to good correlation between molecular tests and histological grade, whether the tests are performed by local pathologists or at a central laboratory (18).

Nowadays, histological grade has been incorporated into multiple algorithms, such as the Nottingham Prognostic Index and Adjuvant! Online™, to determine the prognosis of the breast cancer patients. Furthermore, tumor grade correlates with prognostic molecular subtypes identified by microarray analysis, and microarray-based genomic signatures for histological grading have been devised (18).

1.2.4. MOLECULAR SUBTYPES

With the advent of high-throughput methodologies, valuable tools became available to breast cancer researchers uncover the molecular pathways underpinning carcinogenesis and metastatic proclivity. The establishment of molecular portraits could thus trigger tailored therapy implementation stemmed on pathological signatures, associated with prognosis and response to therapies. The seminal works performed by Sorlie and Perou (20), the Stanford group, based on hierarchical gene cluster analysis, led to the development of a molecular classification of breast cancers that comprised the subtypes luminal (A and B), basal and normal-like, and Her-2. Later, it was attested the prognostic significance of this classification (18, 21-24). The interobserver subjectivity observed with the classic histological classification based on the several flow through working models described above hampered its universal acceptance among the pathologists. Gene expression profiling could be the solution to a global classification of breast cancer, perhaps becoming the gold standard technique, but besides economical issues, the appliance of this technique was precluded by the facts that called into question the validity of this taxonomy (18). Therefore, for risk estimation and choice of adjuvant systemic therapies for patients with operable breast cancers, information on ER, PgR, and Her2-*neu* proteins expressions is still routinely utilized worldwide (24). The intrinsic subtypes can also be defined more simply at the protein expression level by immunohistochemistry for ER, PgR, Her2-*neu*, and myoepithelial/basal markers, eg, cytokeratins (CKs) 5/6, CK17, and CK14 (24, 25).

In luminal A and B subtypes the estrogen receptor is upregulated. The luminal A tumors represent approximately 40% of breast carcinomas (7, 26). The majority is well or moderately differentiated and most occur in postmenopausal women. These cancers are generally slow growing and respond well to hormonal therapy. Conversely, only a small number respond to standard chemotherapy. The gene signature is dominated by dozens of ER regulated genes (27). Luminal B group comprises 15-20% of breast carcinomas and also expresses ER, but generally present higher grade, higher proliferative rate, and often overexpress Her2-*neu* (26, 27). They are sometimes defined as triple positive cancers and compose the major group of ER-positive cancers that are more likely to be associated with lymph node metastases and that may respond to chemotherapy (27). The normal-like tumors representativeness is low and it is not yet clear whether or not this is a specific tumor expression pattern (28). This small group of usually well differentiated ER-positive, Her2-*neu* negative cancers, is characterized by the similarity of their gene expression pattern to normal tissue (27). The basal-like group, which represents 15-20% of breast cancers (26), is notable for the absence of ER, PgR and Her2-*neu* proteins and the expression of typical markers of myoepithelial cells (basal keratins, P-cadherin, p63 or laminin), progenitor cells, or putative stem cells (CKs 5 and 6). They have special genetic and epidemiological features, and generally present high grade and proliferation rate (27). They are associated with an aggressive course, frequent visceral and brain metastases, and poor prognosis. However, approximately 40% will have a pathologic complete response to chemotherapy; cure may be possible in this chemosensitive subgroup (27). Finally, Her2-*neu* positive breast cancer, which comprises 10-15% of breast cancers (27), are ER-negative carcinomas that overexpress the Her2-*neu* protein. In over 90% of Her2-*neu* positive cancers, overexpression is due to amplification of the segment of DNA on 17q21 that includes *ERBB2* (alias: *Her2)* and varying numbers of neighbor genes. This amplicon dominates the gene signature of this group. These cancers are usually poorly differentiated, have a high proliferation rate, are associated with a high frequency of brain metastases and have an intermediate benefit from chemotherapy (27).

1.3. BREAST CANCER TREATMENT

Surgery is the usual first line treatment, being breast conservation surgery the ideal surgical approach. The decision depends upon the initial assessment of tumor size and extent, and the presence of axillary lymph node metastases, which is the most powerful prognostic determinant in primary operable breast cancer (3, 29).

After surgery each patient should have a plan for adjuvant therapies. The adjuvant treatments have been shown to reduce the risk of recurrence and to improve overall survival (29). Postoperative pathological assessment of the surgical specimen should be made according to the pathological TNM staging system (Table 4), and also taking into account several biological tumor characteristics, such as histological type and grade (Tables 1 and 2) and immunohistochemical evaluation of ER, PgR, Ki67 and Her2-*neu*. *ERBB2* (alias *Her2*) gene amplification status should be determined by *in* situ hybridization techniques as chromagenic *in situ* hybridization (CISH) or fluorescent *in* situ hybridization (FISH) if immunohistochemistry results are doubtful (3, 30, 31).

TABLE 4: Staging of breast cancer based on TNM classification [adapted from (12)].

STAGE GROUPING	T	N	M
Stage 0	Tis	N0	M0
Stage I	T1	N0	M0
Stage IIA	T0	N1	M0
	T1	N1	M0
	T2	N0	M0
Stage IIB	T2	N1	M0
	T3	N0	M0
Stage IIIA	T0	N2	M0
	T1	N2	M0
	T2	N2	M0
	T3	N1, N2	M0
Stage IIIB	T4	N0, N1, N2	M0
Stage IIIC	Any T	N3	M0
Stage IV	Any T	Any N	M1

The integration of the clinical parameters into scores (e.g. Nottingham Prognostic Index) that allow to accurately estimate the probability of recurrence and/or death are useful clinical tools to stratify patients and establish the best adjuvant

therapeutic scheme, being radiotherapy the most common adjuvant treatment option (29). Conventional systemic therapy, if the patient's benefit surpasses the risks and side effects, is also offered. According to tumor ER and Her2-*neu* status, endocrine or targeted therapy, respectively, may also be recommended (3).

These combined therapy approaches have been successful, in view of the almost steady state of breast cancer mortality during the past three decades. Although implementation of individualized therapy is now possible, about 60% of all patients with early-stage breast cancer still receive adjuvant chemotherapy, of which only a small proportion, 2-15% of patients, will ultimately derive benefits, all being subjected to the risk of toxic side effects (27). Furthermore, this treatment approach is not cost effective. The hunt for prognostic markers that accurately predict the risk of developing breast cancer or the progression of the disease is therefore far away from being completed.

2. PATHWAYS TO BREAST CANCER

Despite the therapeutic arsenal available, breast cancer is still a public health problem. The cases diagnosed as breast cancer are described as a complex and heterogeneous collection of pathologies of the same anatomical site, originated from the same anatomical structure (i.e., TLDU) but encompassing different pathobiological entities. They are characterized by different risk factors, histological features, and a scope of clinical behaviors and response to treatment, and ultimately disease related morbidity and mortality. These features cannot be determined by anatomical prognostic factors (i.e., tumor size or nodal status), but rather by intrinsic molecular characteristics of the tumors that can be probed with molecular methods (27).

The study of cancer genetics has had an enormous impact in our understanding of the development and progression of breast neoplasias. The high-throughput methodologies applied over the last few years to the analysis of malignant and precursor lesions of the breast were extremely important to bring some light into the pathways of breast carcinogenesis. They provide strong circumstantial evidence that the multitude of pathological phenotypes and diverse behavior are molecularly underpinned by complex patterns of genetic and epigenetic

alterations, which deregulate the control of some genes, and concomitantly the cellular pathways associated with them, destabilizing vital cell processes (32).

Breast carcinogenic pathways remain an incompletely solved conundrum, partly due to the diversity of prognosis, even among patients with similar clinical stages.

2.1. STEP BY STEP TOWARDS BREAST CANCER

The least understood step of breast carcinogenesis is the transition of carcinoma *in situ* to invasive carcinoma. Contrarily to other tumors, such as colon cancer, it has been difficult to understand the molecular events underlying the development of a precursor breast lesion into an overtly malignant phenotype, namely due to methodological obstacles, like the scarceness of samples of precursor lesions and the difficulty to translate *in vitro* to *in vivo* models (19).

Additionally, the identification of specific genetic tumor markers has been difficult. If we look carefully to normal breast tissue function, we encounter intricate and exceedingly complicated pathways that depend on the interplay between luminal, myoepithelial and stromal cells. The same molecular events that allow the normal formation of new ductal branch points and lobules during pregnancy and puberty, namely abrogation of the basement membrane, increased proliferation, escape of growth inhibition, angiogenesis, and invasion of the stroma, may be recapitulated during carcinogenesis (7). Remodeling of the breast, which involves inflammatory and wound healing like tissue reactions, could explain the transient increase in breast cancers during and shortly after pregnancy, since such changes could facilitate the transition of carcinoma *in situ* to invasive cancer (7).

Several models of breast carcinogenesis have been proposed. Wiechamnn *et al* (33) proposed two models: the multistep model or the “theory of linear progression” and the “theory of parallel disease”. The former model supports that tumor progression follows a linear pattern and thus invasive breast cancer develops after breast disease proceeds through sequential stages, from premalignant hyperplastic breast lesion with and without atypia (atypical ductal hyperplasia, atypical lobular hyperplasia and usual ductal hyperplasia), to carcinoma *in situ* (ductal or lobular) and to invasive carcinoma. This model has been supported by molecular and clinical evidence, but the behavior of non

invasive lesions is inconsistent. Fifty percent of ductal carcinoma *in situ* progress to invasive breast cancer, but the range of time of progression from non malignant lesion to an overt malignant phenotype is highly variable (33). In the second model, low grade non invasive lesions are committed to progress to low grade invasive counterparts, and the same correspondence is established between non invasive and invasive high grade lesions. The idea that these two models are mutually exclusive may represent an oversimplification (33).

Breast cancer is a genetic disease in which the major risk factors for its initiation and proliferation are genetic and hormonal, as mentioned above. The sporadic forms are specially influenced by exposition to hormones. In accordance with this evidence, the majority of breast cancers are ER positive and occur in postmenopausal women. Probably these carcinomas arise from ER-expressing luminal cells, which is supported by the phenotype of some precursor lesions, such as atypical hyperplasias, that are more similar to this type of cells. ER-negative carcinomas may arise from ER-negative myoepithelial cells. This would explain why many proteins found in myoepithelial cells are shared by the triple-negative (the standard evaluated markers are unexpressed) or basal-like cancers (7).

From studies addressing genotypic-phenotypic correlations has become apparent that estrogen receptor positive and negative breast cancers are two distinct pathological entities (19). Within ER-positive breast cancer, histological grade and proliferation are strongly associated with the extent, complexity and type of genetic aberrations (19). Lopez-Garcia *et al* (19) proposed two working models based on histological grade, immunohistochemistry and molecular features of breast cancer, as illustrated in Figure 2.

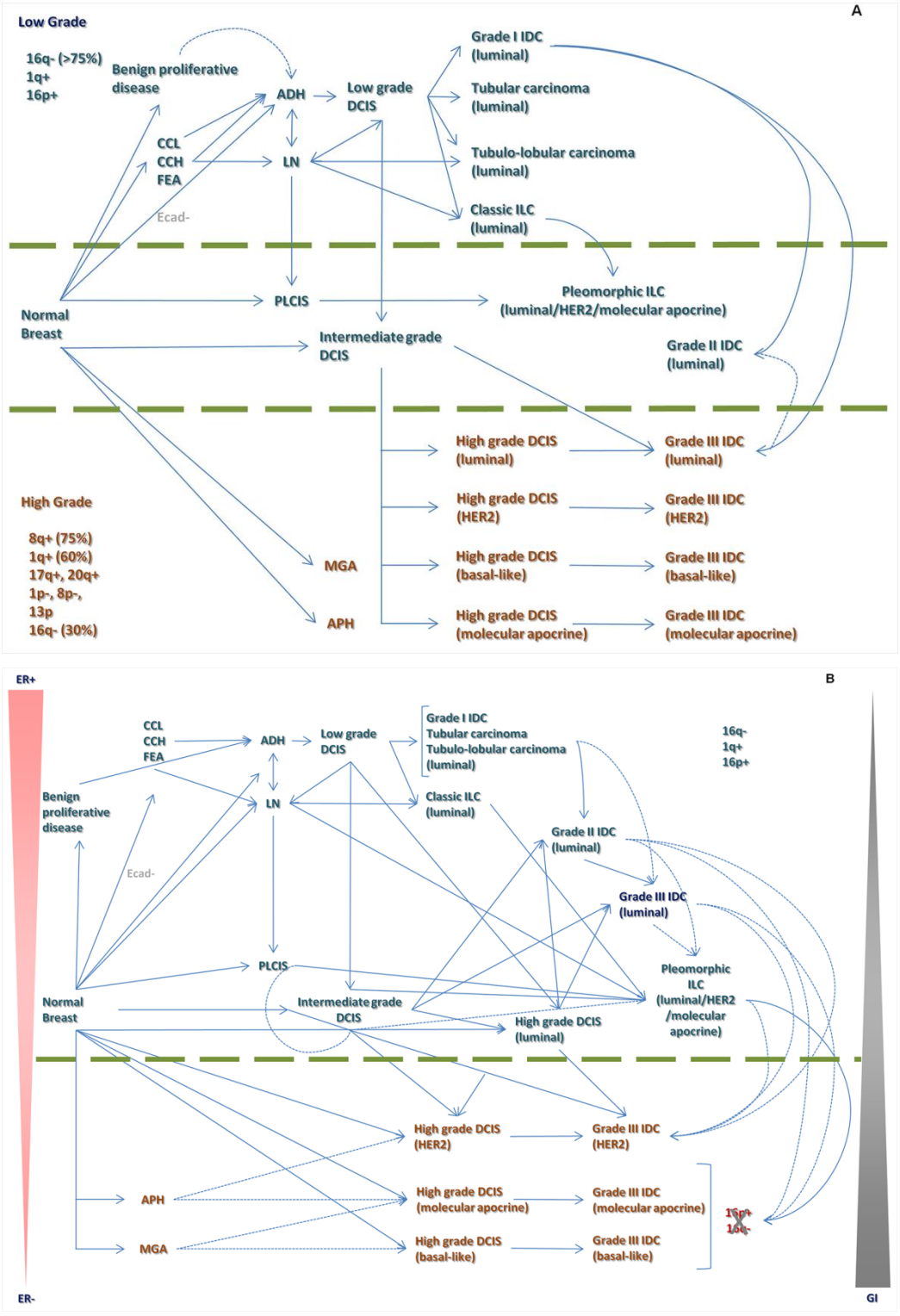

FIGURE 2: Multistep model of breast cancer evolution. **A**) Pathways to low grade and/or high grade forms of breast cancer based on morphological, immunohistochemical and molecular features; connectors drawn with continuous lines represent links between morphological entities which are demonstrated by morphological and/or molecular data; connectors drawn with discontinuous lines represent hypothetical links yet to be demonstrated. **B**) Pathways stemmed from ER status. Note that the two main pathways are defined by the expression of ER and ER-regulated genes. In this model, the ER-positive arm encompasses most of the precursor lesions and a range of invasive

FIGURE 2 (CONT): lesions which may progress from low to high grade due to the acquisition of genetic instability (GI) and accumulation of stochastic genetic events. The ER-negative arm includes ER-negative DCIS and invasive tumors; MGA and APH are proposed as non-obligate precursors of these lesions. ER and genetic instability bars on either side of the image represent the levels of ER expression and genetic instability, respectively. ADH: atypical ductal hyperplasia; APH: atypical apocrine hyperplasia; CCH: columnar cell hyperplasia; CCL: columnar cell lesion; DCIS: ductal carcinoma *in situ*; E-cad: E-cadherin; FEA: flat epithelial atypia; IDC: invasive ductal carcinoma; ILC: invasive lobular carcinoma; LN: lobular neoplasia; MGA: microglandular adenosis; PLCIS: pleomorphic lobular carcinoma *in situ* [extracted from (19)].

2.2. MOLECULAR BASIS OF BREAST CANCER

All cancers carry somatic mutations in their genomes. A subset, known as driver mutations, confer clonal selective advantage on cancer cells and are causally implicated in oncogenesis, and the remainder are considered passenger mutations (34).

When compared to normal cells, neoplastic cells present the so-called “hallmarks of cancer”. These features print to cells special capabilities necessary for cancer initiation and progression, namely the limitless capability of mitosis, self-sufficient growth promoting signals, to induce angiogenesis, the abilities to evade inhibitory growth control and invade surrounding and distant tissues, resistance to apoptosis, reprogramming cellular metabolism and to avoid the immune system. After developing all these competences, the cell is prepared to turn into a neoplastic cell.

2.2.1. ONCOGENES AND TUMOR SUPPRESSOR GENES

Breast cancer develops as a consequence of the accumulation of carcinogenic mutations in the genomes of the evolving neoplastic cells. These mutations range from point mutations and copy number changes affecting only a few nucleotides up to gain or loss of whole chromosomes, or large fractions of chromosomes at a greatly increased rate compared with normal cells. The resulting effects of these aberrations are the activation of cancer promoting genes (oncogenes) and/or the inactivation of genes that protect the cells from malignant transformation (tumor suppressor genes). Another important class of genes, the caretakers, maintain cells’ genomic stability. When these genes are inactivated, cancer may occur, as a

cell loses its ability to maintain the normal mutation rate and therefore the likelihood of arising carcinogenic mutations increases.

Oncogenes overexpression, such as *ERBB2* (35, 36) and *MYC* (37), and loss of tumor suppressor genes, like *TP53* (38) or *PTEN* (39), are proposed to underlie breast cancer initiation and progression. Several mechanisms are involved in gene activation/inactivation and may contribute to breast carcinogenesis (32, 40, 41).

2.2.2. EPIGENETIC ALTERATIONS

Epigenetic alterations have been described as a carcinogenic mechanism in many cancers, including in breast tumorigenesis (42). Cancer genomes as a whole are hypomethylated, but the hypermethylation of CpG islands in the neighborhood of promoters leads to gene silencing, which is a common mechanism for inactivation of tumor suppressor genes.

2.2.3. ANEUPLOIDY AND CHROMOSOMAL ALTERATIONS

Aneuploidy is defined as the gain and/or loss of whole chromosomes and is almost ubiquitously found in neoplastic lesions. It is frequently caused by chromosomal instability defined as the inability of cells to properly divide the chromosomal set into the daughter cells (43). Aneuploidy may contribute to carcinogenesis by the inactivation of tumor suppressor genes, by physical elimination of a gene or its regulatory regions, or by the activation of oncogenes, due to a dosage effect (43). This phenomenon can be related to a gene in particular, but it is usually associated to the disequilibrium of a gene set.

There are several reports in the literature using conventional cytogenetics and comparative genomic hybridization (CGH) showing recurrent chromosomal alterations in breast cancer specimens, namely gain of 1q, 8q, 16p, 17q, and 20q and loss of 16q and 17p (44-47). However, the candidate genes localized in these regions have not yet been identified.

Genomic amplifications are the selective increase in copies of DNA. They can involve a single gene encompassing a few kilobases or multiple genes, neighboring or located in distant genomic locations. These events have classically been related to cytogenetic features, such as double-minutes, self replicating

extra-chromosomal elements, or homogeneously staining regions, where multiple copies of a single or several genomic regions are incorporated into a chromosome (48). On the other hand, homozigous deletions that commonly occur in neoplastic cells are thought to primarily inactivate tumor suppressor genes. Nevertheless, the deletion mechanism can also result in a fusion gene, such as the *TMPRSS2–ERG* in prostate cancer (49). This kind of events may be associated with the presence of fragile sites. The occurrence of fragile sites across the genome can also be associated with recurrent homozigous deletions in cancer cells (50).

Another mechanism for oncogene activation is translocation, which is the most common mechanism of origin of fusion genes, whose activity is different from the ones that originated them. Any badly repaired event that generated double strand DNA breaks can be responsible for the origin of a fusion transcript. The discovery of translocations involving the ETS-family members in prostate cancer, as well as genes like *ALK* in lung cancer (51), suggests that fusion genes may play a more prominent role in epithelial carcinogenesis than previously thought. Fusion genes have rarely been described in breast cancer cell lines and tumor samples, but rare breast cancer subtypes are characterized by recurrent fusion transcripts, namely the secretory breast carcinoma with the *ETV6-NTRK3* transcript and the adenoid cystic carcinoma of the breast with the fusion transcript *MYB-NFIB* (52). Recently, rare but recurrent rearrangements of the NOTCH and MAST family genes (e.g. *NOTCH1-NUP214*) (53), as well as the recurrent *RPS6KB1-VMP1* fusion (54), were also described in breast cancer.

2.2.4. ETS GENES

The E26 transformation-specific (ETS) family of genes is restricted to metazoans and thus represents an evolutionarily recent class of transcription factors (55, 56). These genes regulate a spectrum of normal biological activities, namely cell differentiation, development, homeostasis, proliferation, and apoptosis. It is one of the largest transcription factor families, consisting of 28 ETS genes in humans (55, 56). The ETS family is defined by the ETS domain, a highly conserved DNA-binding domain (DBD), which is a purine-rich core sequence localized within the enhancer or in the promoter regions of downstream genes (Figure 3) (55, 56).

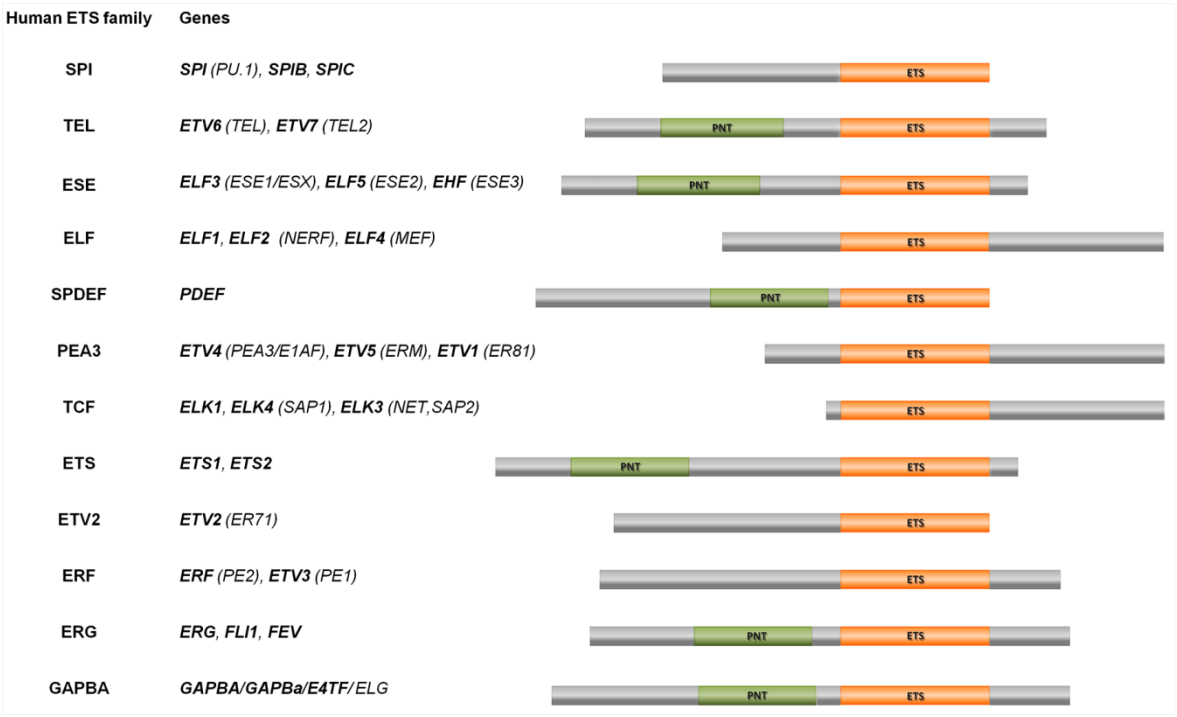

FIGURE 3: Structural and functional domains of the ETS family of transcription factors. Nomenclature and domain organization of the 28 ETS paralogous human ETS proteins [grouped according (57)]. The HUGO nomenclature for all ETS proteins and genes are highlighted at bold, and the alternative names are also provided. Multiple protein products of ETS proteins are synthesized by alternative splicing/start sites, a single polypeptide was chosen arbitrarily. Boxes identifying ETS domains are represented in orange and the pointed domain (PNT) in green. Other additional regions involved in several biological functions were not represented [adapted from (57)].

The ETS genes also play important roles in oncogenesis. Deregulated expression or genetic alterations of ETS genes are thought to represent a significant role in the development of human neoplasias (56). Chromosomal translocations involving ETS genes have been reported in hematological and soft tissue malignancies and result in an inappropriate expression level or expression as fusion proteins (56). The role of this family of genes in epithelial cancers (carcinomas) was less established until recently, when *ERG*, *ETV1*, *ETV4*, *ETV5* and *FLI1* were shown to be fused with *TMPRSS2*, or other 5' fusion partners, in a significant proportion of prostate carcinomas (58-60). These fusion oncogenes are thought to increase ETS expression in response to androgen, leading to prostate epithelial cell transformation (59).

Secretory breast cancers, a very unusual histological breast cancer type (represents less than 1% of infiltrating breast carcinomas) (61), presents a

recurrent chromosomal translocation, t(12;15)(p13;q25) (62). This cytogenetic finding had previously been described in congenital fibrosarcoma, congenital mesoblastic nephroma, and acute myeloid leukemia (63-65). The translocation generates a gene fusion encoding the dimerization domain of the transcription factor ETV6 and the tyrosine kynase domain of the NTRK3 (62). The protein ETV6-NTRK3 functions as a potent chimeric protein tyrosine kinase, with *in vivo* and *in vitro* transforming activity, which leads to constitutive activation of the RAS-MAPK mitogenic pathway and the PI3K-AKT pathway (66). This unique ability may be crucial for its carcinogenic capability. Additionally, transcriptome studies have also indicated that many of the molecular changes associated with breast cancer are mediated by altered function of transcription factors, namely ETS genes (67-69).

ETS genes are also involved in healthy breast tissue proliferation and differentiation. Studies performed with mice suggest relevant roles for *ETV4*, *ETV5* and *ETV1* genes in mammary embryonic organogenesis and in postnatal tissue differentiation (70).

AIMS

AIMS

The main goals of this work were:

1) To identify the pattern of genome alterations in a large series of breast carcinomas;

2) To characterize the pattern of gene expression alterations of the ETS family of transcription factors;

3) To determine the role of genomic copy number gains and amplifications on overexpression of the ETS proteins;

4) To uncover target genes affected by altered expression of ETS transcription factors;

5) To establish the prognostic value of gene expression alterations of ETS transcription factors in breast cancer**.**

MATERIAL AND METHODS

MATERIAL AND METHODS

1. PATIENTS AND TUMOR SPECIMENS

The tumor samples used in this study were collected from breast cancer patients treated at the Portuguese Oncology Institute-Porto, who underwent potentially curative resection between 1999 and 2001. After pathological examination tumors were snapped frozen in liquid nitrogen and subsequently stored at -80ºC until the molecular analysis were performed. A total of 141 specimens were evaluated during this study. We selected the first consecutive cases, with follow-up data of 10 years, of this series of fresh-frozen breast carcinoma samples. The clinicopathological characteristics were obtained for all patients and their clinical course revised by two independent oncologists. This data is summarized in Table 5.

Paired frozen and paraffin-embedded samples were re-evaluated to assure the representativeness of cancer cells (>75% of tumor cells). Fifteen 15µm thick tissue sections of frozen tissue were used for DNA and RNA isolations. Sections of paraffin embedded tissue were used as described below to analyze ER, PgR and CKs 5/6 and 14 by immunohistochemistry, as well as copy number changes of *ERBB2* and particular ETS genes by FISH.

2. IMMUNOHISTOCHEMICAL CHARACTERIZATION

The breast carcinomas were retrospectively re-evaluated independently by two pathologists for morphological and immunohistochemistry characterization. This step ensured that all breast cancer specimens were reclassified using the standard methods in use nowadays. Commercially available antibodies were used for ER (Clone 6F11; Novocastra, Newcastle, UK) and PgR (Clone 16; Novocastra, Newcastle, UK), as well as for CKs 5/6 (Clones: D5 & 16B4; Cell Marque; Rocklin, CA, USA) and 14 (Clone SP53; Cell Marque; Rocklin, CA, USA).

TABLE 5: Clinicopathological characterization of 141 breast cancer patients[a].

CLINICOPATHOLOGICAL CHARACTERISTICS	
Age at diagnosis (mean±SE)	59.3 ±1.2
Follow-up (mean±SE; months)	88.9±3.5
Histological grade (Elston-Ellis)[b]	
1	8.5%
2	48.9%
3	42.6%
Histological type	
Ductal	65.2%
Lobular	9.2%
Medular	1.4%
Others	24.1%
Pathological stage[b]	
I	12.1%
IIA	36.2%
IIB	18.4%
IIIA	10.6%
IIIB	9.9%
IIIC	7.8%
IV	5.0%
Carcinoma in situ*	
Without	32.6%
Not extensive	37.6%
Extensive	28.4%
Family history of cancer*	34.0%
Family history of breast cancer*	13.4%
Chemotherapy*[a]	
Neoadjuvant	11.3%
Adjuvant	56.7%
Adjuvant radiotherapy*	70.9%
Follow up status*	
Alive disease free	63.8%
Alive with disease	5.7%
Dead disease free	21.3%
Dead with disease	7.8%

Legend: [a]All patients were diagnosed before anti-ERBB2 agents became available. [b]Based on stage grouping defined by World Health Organization (12). *Data not available for all patients.

The tissue slides were deparaffinized by rinsing with xylol and rehydrated by passing through graded alcohols (absolute ethanol, 90%, 80%, 70%, 50%). Antigen retrieval was performed with citratre buffer. Endogenous peroxidase activity was blocked by incubating the slides for 20min in 0.3% $H_2O_{2\ (aq)}$. After washing the slides in water and PBS/0.05% Tween 20 solution, they were incubated with normal serum (Vectastain Universal Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA) 1/100 in PBS-Bovine Serum Albumine (BSA) 1%, at room temperature for 20min in a humid chamber. After incubation, the respective primary antibodies were added at an optimal dilution for each antibody in PBS-BSA 1% solution, and the slides were incubated overnight at 4°C in a humid chamber. The slides were then rinsed in PBS/0.05% Tween 20 solution and bound antibody was detected by applying biotinylated secondary antibody and ABC reagent from the Vectastain Universal Elite ABC kit (Vector Laboratories**,** Burlingame, CA, USA**)**. The slides were washed in PBS and developed with a peroxidase substrate solution [0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB 3,3'-Diaminobenzidine, Sigma-Aldrich, Steinheim, Germany) and 0.01% H_2O_2 in PBS] during 7min. Counterstaining was then performed with hematoxylin (Harris Modified Hematoxylin Stain, Fisher Scientific, Fair Lawn, NJ, USA) for 30s and washed for 10min in H_2O. Slides were mounted with Entellan (Merck KGaA, Darmstadt, Germany). For each antibody appropriate positive and negative breast cancer controls were used. The control samples used were breast cancer tumor specimens analyzed by immunohistochemistry and revaluated by FISH. Sections were scored as negative when <10% and as positive when ≥10% of stained cells were present.

3. *ERBB2* GENE CHARACTERIZATION

ERBB2 status was evaluated by FISH using a dual color commercial probe (Poseidon, Kreatech Diagnostics, Amsterdam, The Netherlands), targeting the chromosome 17 centromere and the *ERBB2* gene. The centromeric probe is directly labeled with Platinum*Bright*495 and produce green signals; the *ERBB2* gene probe is labeled with Platinum*Bright*550 and present red signals The *ERBB2* status classification followed international consensus guidelines (31). The cases not analyzable by FISH were scored according to the CGH results, as CGH and FISH present an 82% concordance regarding *ERBB2* status (71). One hundred

and twenty six cases were classified by FISH and 15 cases were classified according to the CGH results. From each of the paraffin-embedded samples, four-micron thick sections of a representative tissue block were cut onto SuperFrost Plus Adhesion slides (Menzel-Glaser, Braunschweig, Germany). Slides were deparaffinized in two series of xylol followed by two series of ethanol, rinsed in 2xSSC, and placed in a solution of NaSCN 1M at 80ºC. Tissues were then digested with pepsin, after which slides were rinsed in 2xSSC and dehydrated in an ethanol series. The probes were applied onto each sample and slides were placed in a Hybrite denaturation/hybridization system and co-denatured. Hybridization took place overnight, followed by post-hybridization washes in 2xSSC/0.5% Igepal and 2xSSC/0.1% Igepal, after which slides were counterstained with DAPI. Fluorescent images corresponding to each fluorochrome and DAPI were sequentially captured with a Cohu 4900 CCD camera, using an automated filter wheel coupled to a Zeiss Axioplan fluorescence microscope and a CytoVision system. Only intact, non-overlapping nuclei were scored.

4. COMPARATIVE GENOMIC HYBRIDIZATION

Our initial goal was to evaluate the global pattern of chromosomal alterations in this series of the breast carcinomas. The chosen methodology was CGH, a technique that provides an overview of the changes in DNA copy number across the whole genome (Figure 4).

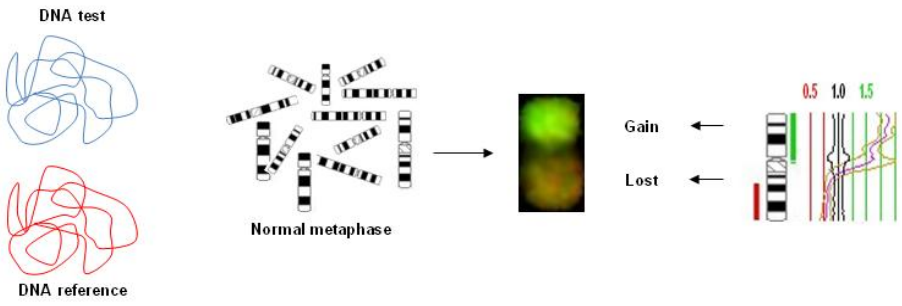

FIGURE 4: Diagram representing the CGH technique. Tumor and reference samples DNA are each labelled with specific fluorochromes, hybridized onto normal metaphases and the fluorescence differences evaluated by a specific software.

Tumor samples were digested with proteinase K (20mg/mL) in a lyses buffer [NaCl 75mM, 25mM EDTA (pH 8) and SDS (10%)], at 55ºC until complete digestion.

DNA was extracted with phenol-chlorophorm (pH 8), precipitated with isopropanol and ammonium acetate, reprecipitated in ethanol and eluted in water. DNA quality was evaluated by agarose gel (0.8%; w/v) electrophoresis and the quantity and purity were measured in Nanodrop (ND-1000 Spectrophotometer, Wilmington, USA).

The CGH procedure described by Kallioniemi *et al* [12] was performed with modifications previously described in Teixeira *et al* [8] and Ribeiro *et al* [13]. Briefly, test DNA (tumor sample) and reference DNA (genomic DNA extracted from blood lymphocytes from healthy donors) were differentially labeled with green and red fluorescent dyes, respectively, by nick translation using fluorochrome-conjugated nucleotides, resulting in fragment lengths between 300–2000bp. Test and reference DNA were mixed in a 1:1 proportion with unlabeled human Cot-1 DNA (Invitrogen, Life Technologies, Carlstad CA, USA), ethanol precipitated, dried, and dissolved in hybridization buffer. DNA probes and commercially available normal metaphase chromosomes, prepared from normal lymphocyte cultures, were denatured following by co-hybridization (2-3 days in humidified chamber at 37ºC). After a series of washes, the slides were mounted in an antifade solution with DAPI. Using a Cytovision system with software version 3.9 (Applied Imaging, Santa Clara, CA, USA), chromosomes were identified based on their inverted DAPI appearance and the relative hybridization signal intensity determined along each chromosome. Data obtained from ten cells were combined to generate average ratio profiles with 99% confidence intervals (CI) for each chromosome. Ten cells of each of ten normal versus normal hybridizations were used to establish the normal ratio profile with 99% CI [14]. Copy number gains and losses were scored whenever the test and the reference 99% CI did not overlap. Amplifications were scored whenever the 99% CI of the fluorescence ratio was above 1.5 in part of a given chromosome arm. The description of the CGH copy number changes followed the guidelines suggested by the ISCN [15].

5. COPY NUMBER EVALUATION OF THE *ETV3*, *ELF3* AND *ELK4* GENES

Based on the results obtained after CGH, we evaluated the copy number of the ETS genes on 1q. *Locus*-specific probes derived from bacterial artificial chromosomes (BACs) were selected according to physical and genetic mapping

data on chromosomes reported in the Human Genome Browser at the University of California, Santa Cruz website (http://genome.ucsc.edu/). BAC clones targeting *ETV3* (CTD3076J23A), *ELF3* (RP11465N4B and CTD2545E14B) and *ELK4* (CTD2218H7B) were selected to cover each of the three genes and obtained from the BACPAC Resources Center (Oakland, California, USA). Adequate mapping and probe specificity of all BAC clones was confirmed by hybridization onto normal human metaphases. DNA was extracted using the Plasmid DNA Purification Kit (MACHEREY-NAGEL GmbH & Co. KG, Duren, Germany) and amplified using the GenomiPhi V2 DNA Amplification Kit (WGA kit, GE, Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. After a 5min pre-treatment at 90ºC, BAC DNA was labeled with SpectrumGreen or SpectrumRed (Abbott Laboratories, IL, USA) conjugated nucleotides in nick translation reactions using the same protocol as described above for CGH. Seven hundred ng of each labeled BAC probe was then mixed with 30µg unlabeled Cot-1 DNA (Invitrogen, Life Technologies), ethanol precipitated, dried, and dissolved in hybridization buffer (Abbott Laboratories, IL, USA). We selected cases for FISH validation according to the CGH findings, including 10 cases without 1q alterations, 10 with 1q amplifications and 10 with 1q gain. The FISH technique was performed as described above.

6. GENE EXPRESSION

Gene expression of a set of ETS genes was analyzed by quantitative real-time PCR (qRT-PCR) (Table 6). Our choice was based on previous published results (69) and on the CGH results. We also choose a panel of potential ETS target genes, namely *CRISP3* and *MYC*, which have been described as possible ETS target genes in hormone dependent tumors such as breast and prostate cancers (37, 60, 72-76).

TABLE 6: Group of ETS genes and the possible ETS target genes selected for gene expression analysis.

GENE	*LOCUS*	ASSAY	AMPLICON LENGTH (bp)
ETV3	1q21~q23	Hs00366497_m1[*]	66
ELF3	1q32.2	Hs00231786_m1*	74
ELK4	1q32	Hs01111811_m1*	61
ETV5	3q28	Hs00231790_m1*	70
ETV1	7p21.3	Hs00951941_m1*	110
ETS1	11q23.3	Hs00901425_m1*	67
FLI1	11q24.1~q24.3	Hs00956711_m1*	157
ETV4	17q21	Hs00385910_m1*	95
ETS2	21q22.2	Hs00232009_m1*	93
ERG	21q22.3	Hs01554635_m1*	104
CRISP3	6p12.3	Hs00195988_m1*	111
MYC	8q24.21	Hs00153408_m1*	107
HPRT1[b]	Xq26.1	Hs99999909_m1*	100
18S[b]	-[c]	Hs99999901_s1[a, d]	187

Legend: *"_m": indicates an assay whose probe spans an exon junction and will not detect genomic DNA. [a]"_s": indicates an assay whose probes and primers are designed within a single exon and, hence, will detect genomic DNA. [b]The two normalizing genes used. [c]Multiple *loci* in distinct chromosomes. [d]According to Applied Biosystems, Life Technologies based on assay re-evaluation in August 2010 with NCBI Entrez Gene annotations, this assay may detect transcript(s) from off target gene. Our work was complete before this date.

Total RNA was extracted from frozen breast tumor samples using Trizol® Reagent (Invitrogen, Life Technologies) and PureLink®RNA Mini Kit (Invitrogen, Life Technologies) as per the manufacturer's instructions. All RNA samples were digested with TURBO™ DNAse (Ambion, Applied Biosystems, Life Technologies, Rockville, MD), according to the manufacturer's instruction, to improve the purity of the RNA and avoid genomic DNA contamination in subsequent RT-PCR. The quality of the RNA was checked by electrophoresis on 1% (w/v) agarose gel and the quantity and the purity ratio were evaluated in a Nanodrop 1000 (ND-1000 Spectrophotometer). Samples showing intact 28S and 18S rRNA bands were selected for the analysis.

In order to improve the amount of total RNA and obtain cDNA, we used Transplex®, an Whole Transcriptome Amplification (WTA) method (Sigma-Aldrich, Saint Louis, Missouri, USA) (77). Briefly, for the library preparation, to 5–300ng of total RNA, we added 2.5µL WTA Library Synthesis Buffer and 2.5µL WTA Library

Stabilization Solution and completed with Nuclease-free water for a total volume of 24µL. After incubation at 70°C for 5min, the reaction was immediately cooled on ice and then we added 1µL of WTA Library Synthesis Enzyme and incubated in thermal cycler (24°C for 15min; 42°C for 2h; 95°C for 5min). The reaction was immediately chilled on ice. Then we proceeded to library amplification: after preparing a WTA Amplification Mix (300µL Nuclease-free water; 37.5µL WTA Amplification Master Mix; 7.5µL dNTP Mix; 12.5 units of antibody inactivated hot-start Taq DNA Polymerase), we added 70µL of this mixture to each 5µL aliquot of the library, and incubated in thermal cycler using the following parameters: 95°C for 3min; 94°C for 20s, and 65°C for 5min (20 cycles). Then we purified the cDNA with a commercial kit (Quiagen, West Sussex, UK).

Quantitative PCR reactions were performed on a ABI Prism 7900 HT sequence detection system, using Taq®Man Low-Density Array (TLDA) (Applied Biosystems, Life Technologies). The primers and probes of each assay targeting the elected ETS genes and their potential target genes and the two normalizing housekeeping genes (*18S* and *HPRT*) were preloaded and dried onto designated duplicate wells. In the literature there is no consensus on which is the best reference gene for gene expression analyzes of breast carcinomas (78, 79), so we decided to use the median gene expression results after normalization with each reference gene to minimize sample variability and to increase the accuracy and resolution of gene expression normalization.

7. STATISTICAL ANALYSIS

The non-parametric Mann-Whitney or Kruskal Wallis tests were used to compare RNA expression levels of ETS genes in different sample groups. To assess associations of continuous variables, the Spearman non-parametric correlation test (rs) was used. Kaplan Meyer curves and log rank tests were used to evaluate differences between disease survival and breast cancer molecular subtypes. Correlations between 1q gain and breast cancer molecular subtypes were evaluated using the Chi square test. A *p*-value smaller than 0.05 was considered statistically significant. Statistical analyses were performed using the Statistical Package for Social Sciences software, version 19.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

RESULTS

1. *ERBB2* STATUS AND IMMUNOHISTOCHEMISTRY

Ninety six cases were considered negative (68.1%) and 45 positive (31.9%) for *ERBB2* amplification (Figure 5).

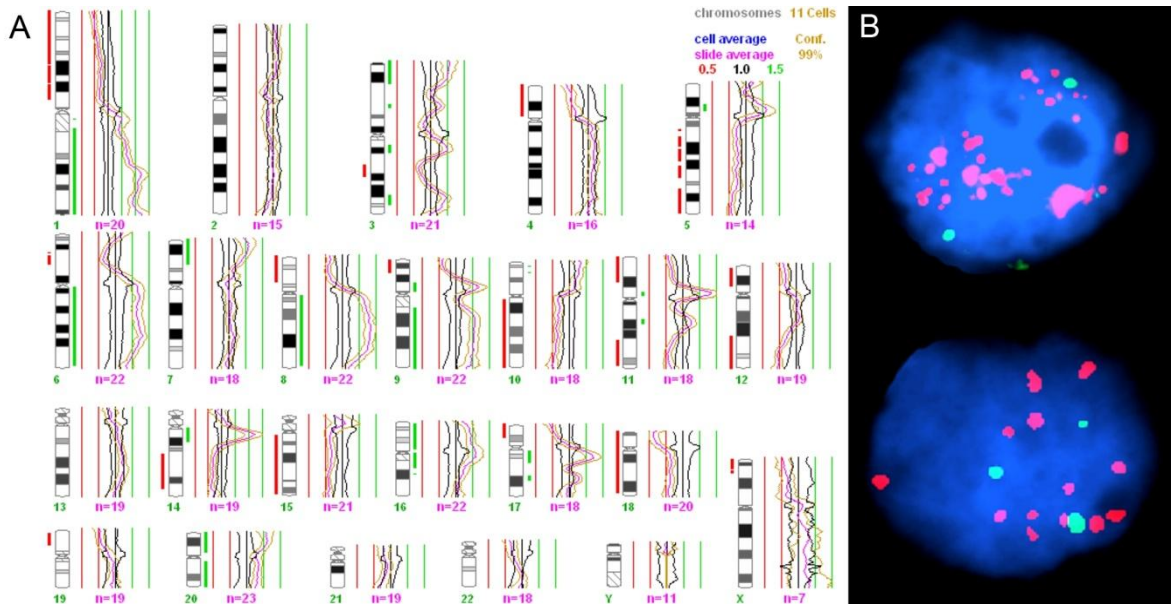

FIGURE 5: A) Comparative genomic hybridization of a breast carcinoma with several copy number changes, showing the most common chromosomal gains observed, namely 1q (where *ETV3*, *ELF3* and *ELK4* are located), 8q (where *MYC* is located at 8q24), and 17q (where *ERBB2* is located at 17q12). Green bars to the right and red bars to the left of the chromosome ideograms indicate copy number gains and losses, respectively. **B**) Interphase nuclei with *ERBB2* amplification (red; control in green).

Regarding the hormone receptor status, 70.2% (99 cases) and 58.9% (83 cases) were considered ER and PgR positive, respectively. Thirty cases (22.9%) were hormonal receptors negative (both ER and PgR), 81 cases (61.8%) were positive for both hormonal receptors, and 20 cases (15.2%) were positive for one of them (18 ER positive/PgR negative; two RE negative/PgR positive). Regarding cytokeratin expression, 32 cases (25.6%) were considered positive, 93 (66%) were negative, and 16 were not analyzable for CKs 5/6 expression, with the findings for cytokeratin 14 being 15 (10.6%), 113 (80.1%), and 13, respectively (Table 7).

TABLE 7: Description of CGH results, immunohistochemistry evaluation for ER, PgR and cytokeratin markers and molecular classification of 141 breast carcinomas.

CASE	CGH FINDINGS	ER[a]	PgR[a]	ERBB2[b]	CKs 5/6[a]	CK14[a]	MOLECULAR CLASSIFICATION
1	rev ish enh(2p14p16,4q28,8q11q24,11p12p15,17q21q25,20q11q13), amp(8q22q24)	+	+	-	+	-	Luminal A
2	rev ish enh(1p32p35,1q12q24,2p23p24,3q12q29,5p13p15,6p12p24,6q23q27,8p11p12,8q13q21,8q22q24, 9p13p23,10q21q23,11q14q24,12p11p13,12q14q15,16p12p13,18p11,18q21q22,20p11p13,20q11,21q22), dim(4q31q35,5q12q35,8p21p22,10p11p15,10q24q26,13q14q33,19p13,22q11q13),amp(3q21q25,8q24,10q22)	+	+	-	-	-	Luminal A
3	rev ish enh(1q31q42,3q21,3q27q28,16p12p13,17q23q25),dim(3p21,8p23,11q22q24,16q12q24)	+	+	-	-	-	Luminal A
4	rev ish enh(17q23q25),dim(16q11q24,17p11p13)	+	-	-	+	-	Luminal A
5	rev ish enh(8p22,8q11q24,16p12p13),dim(1p36,16q11q24)	+	+	-	+	-	Luminal A
6	rev ish enh(1p13,8p11p21,10p12p14,11p11p15,17q23q24,19q13,20q11q13),dim(1p31,3p13p21, 6q13q22,9p12p24,9q12q34,10q11q26,11q23q25,14q32,17p11p13),amp(20q12q13)	+	+	-	-	?	Luminal A
7	rev ish enh(8p12,8q11q24),dim(6q21q27,16q11q24,17p12p13)	-	+	-	-	-	Luminal A
8	rev ish enh(1p13p21,1q21q24,1q32q43,2p11p21,2q22q31,3p22p26,3q21q29,4q31q34,6p21p25,6q12q23, 7q11q36,8q22q24,9p13p24,9q22q34,10p11p15,13q21q34,15q24q25,18p11,20p11p13),dim(Xp21p22,5q32q33, 6q25q27,8p21p23,12p11p13,14q31q32,15q21q22,16p12p13,16q12q23,18q12q23),amp(1q41q43,9p22p24, 10p12p15)	-	-	-	-	+	Basal-like
9	rev ish enh(17q21q24),dim(17p12p13)	+	+	+	-	-	Luminal B
10	rev ish enh(1q12q42)	+	+	-	?	-	Luminal A
11	No alterations	+	-	-	-	-	Luminal A
12	rev ish enh(1q21q43,8p23q24,11q13),dim(9q12q34,11q14q25,16q12q24,17p12p13)	+	+	-	-	-	Luminal A
13	rev ish enh(8p21q24,16p11p13),dim(8p21p23,16q12q24),amp(8p11q24)	+	+	-	-	-	Luminal A
14	rev ish enh(11q13q21,17q21q24,20q11q13),dim(4p13p16,8p21p23,17p12p13,18p11,18q11q21)	+	-	+	-	-	Luminal B
15	rev ish enh(1q12q43),dim(13q12q34,16q12q24,17p11p13)	+	+	+	-	-	Luminal B
16	rev ish enh(1p31p35,1q21q43,2q34q37,8q11q24,9p12q13,9q34,12q13q15,16p11p13,20q11q13,22q11q13), dim(4q25q34,7q33q36,8p12p23,15q22,16q12q24,18q21q22,19q13,21q21),amp(1p32,8q21q24,20q12)	+	+	+	-	-	Luminal B
17	rev ish enh(8q12q24,10q22q23,15q22q25)	+	+	-	-	+	Luminal A
18	rev ish enh(1q12q43),dim(7q22q32,16q11q24)	+	+	-	+	-	Luminal A
19	rev ish enh(11p12p15,11q13q14)	+	-	+	-	-	Luminal B
20	rev ish enh(1q12q42,16p11p12),dim(16q12q24,22q13)	+	+	-	-	-	Luminal A
21	rev ish enh(8p11p21,8p23,8q24,19q13),amp(8p12p21,19q13)	+	+	-	-	-	Luminal A

CASE	CGH FINDINGS	ER[a]	PgR[a]	ERBB2[b]	CKs 5/6[a]	CK14[a]	MOLECULAR CLASSIFICATION
22	rev ish enh(7,8p21q11,14q24q32,18q11q23,22q13),dim(2p22p23,2q21q31,2q34q37,6q13q22,8p21p23, 11q21q25,13q12q34,17p11p13,17q21),amp(8p12p21,18q12q22)	+	+	+	-	-	Luminal B
23	rev ish enh(1q21q43,6p12p25,7p13p21,7q11,8q24,9q21q34,10p11p15,10q21q22,10q26,12p12p13,12q13q24, 14q13q32,16p11p13,20),dim(1p21p36,7q33q36,8p21p23,9p22p24,10q24,13q13q34,16q12q24,17p12p13, 17q11),amp(1q31q43)	+	+	-	-	-	Luminal A
24	rev ish enh(1q21q42,2p21p23,3p21p25,3q12q27,6p12p21,6q13q23,7p13,7q22q36,8p12q24,9p13q21, 10q21q22,14q11q32,15q24q26,16p12p13,17q23q24,18p11,18q12,18q22q23,20q11q13),dim(1p13p21,4p16, 4q22q26,5p15,5q14q31,6q25q27,8p21p23,9q31q34,10q24q26,11p11p15,11q13q24,12p11p12,12q12q24, 13q13q34,15q21q22,16q24,19p13,21q21q22),amp(8q11q24)	-	-	-	?	+	Basal-like
25	rev ish enh(1p21p31,2q11,11p15,14q23q24,17q11q25,20q11q13),dim(1p36,3p14p21,11q22q24,18q21q22), amp(1p31,17q12,17q23q24,20q12q13)	+	-	+	-	-	Luminal B
26	rev ish enh(1q21q43,11q13,12q13q14,16p11p13,17q12q21,19q13,20), dim(6p21q27,9p13p21,11q23q25,15q11q25,16q12q24,21q22,22q13),amp(1q21q42)	+	+	-	-	-	Luminal A
27	No alterations	-	-	-	-	-	Basal-like*
28	rev ish enh(1q22q42,2q22q37,3p12p13,3q12,6p22,6q21q27,7p21p22,7q31q36,8q12q24,9p22p24,10p13p15, 11p12p15,12p13,12q21q24,13q31q34,16p13,16q12q23,17q25,18q12,20q11q13),dim(1p36,4p13p16,4q31q35, 5q22q23,5q33,8p21p23,13q13,17q21,18p11)	-	-	+	+	+	ERBB2 positive
29	No alterations	+	+	-	-	-	Luminal A
30	rev ish enh(6p21p24,8q11q24,11p11p15,12p11p13,12q12q24,16p11p13,21q21q22), dim(11q22q25,16q12q24,22q11q13)	+	+	-	-	-	Luminal A
31	rev ish enh(1p31p32,1q12q43,8q11q24,10p11p15,22q13),dim(Xp11p22,Xq22q27,1p13p22, 1p36,8p12p23,10q25q26,11p11p15,11q22q25,13q21q33,14q24q31,15q11q25,16q12q24,17p12p13), amp(1p31,1q21q42)	+	+	-	-	-	Luminal A
32	rev ish enh(1p33p34,1q21,1q32q41,2p13p24,2q37,3p21p26,3q21,4p16,6p21p22,6q25q27,7q36,8q11q24, 9q31q34,10q24q26,11q13q14,11q23q25,12p12p13,12q24,13q21q34,15q15q26,16p11p13,17q23q25,18q11q23, 19p13,19q13,20q11q13),dim(4q12q35,5p13p14,5q13q35,7p13,8p12p23,11p11p15,12q12q13,14q11q31, 16q12q24,17q11q21,18p11,20p11p13),amp(8q13q24,12p13,13q21q34,18q12q21)	-	-	-	-	+	Basal-like
33	rev ish enh(1q21q42,2p13,4q35,5p15,5q13q21,8q11q24,9p21p23,9q22q34,10p12p15,10q21q23,13q12, 16p12p13,17q12q21,20q11q13),dim(3q11q12,8p21p23,11q22,13q14q21,13q31q34,18p11),amp(8q21q24)	-	-	+	+	-	ERBB2 positive
34	rev ish enh(1q22q42,2p13,2q31,8q13q24,9q34,19p13,21q22),dim(1p36,6q13q16,8p21p22,13q21q33)	-	-	+	+	-	ERBB2 positive
35	rev ish enh(1q12q42),dim(6p21,11q22q23,16q12q24,17p12p13,17q11q25)	+	-	-	+	-	Luminal A
36	rev ish enh(1q21q43)	+	+	-	-	-	Luminal A
37	rev ish enh(17q21q25),dim(8p21)	+	-	+	-	-	Luminal B
38	rev ish enh(1p34p36,1q21,9q34,10q26,11q13,15q12q15,15q22q24,17p13,17q11q21,17q25,19p13,19q13, 20q12q13,22q11q13)	+	-	+	-	-	Luminal B
39	rev ish enh(1q12q42,17p12q21,19p13q13),dim(6q21q27,16q12q24)	+	+	-	-	-	Luminal A
40	rev ish enh(11q13),dim(6q13q16,6q22,6q25q27,8p21p22,11q23,13q21)	?	?	-	?	?	Unclassifiable
41	rev ish enh(9p22p23,11q13q21),dim(Xp22,8p21p22)	-	-	-	+	-	Basal-like
42	rev ish enh(9q34,12p13,16p11p13,),dim(3p13p24,10q22q25,16q24,17p13)	+	+	-	-	-	Luminal A
43	rev ish enh(1q21q43,8p11p23,8q11q24,16p11p13,17q12q25),dim(7q31q35,14q24q32,16q12q24,17p12p13, 21q21q22,22q11q13),amp(1q21q41)	+	+	+	-	-	Luminal B

CASE	CGH FINDINGS	ER[a]	PgR[a]	ERBB2[b]	CKs 5/6[a]	CK14[a]	MOLECULAR CLASSIFICATION
44	rev ish enh(1q22q43,3q26,5q31q33),dim(11q24)	?	?	-	?	?	Unclassifiable
45	rev ish enh(1q12q21,1q25q43,4p13p16,4q21q24,4q26,4q31,5p13p15,8p21q24,17q11q25),dim(3p21p25,11p15, 11q23q25,13q14q34,15q15q25),amp(8q11q22,8q22q24,17q12q21,17q23q24)	-	-	+	-	-	ERBB2 positive
46	rev ish enh(Xp21p22,1p34,1q12q43,3p22p26,3q21,5p14p15,8q12q24,9p23p24,10p11p15,11p12p15,18q12q22), dim(12q24),amp(8q24)	-	-	-	-	+	Basal-like
47	rev ish enh(1q12q43,20p12p13),dim(16q12q24,21q11q22)	+	+	-	-	-	Luminal A
48	rev ish enh(1q21q43,5p13p14,7p13p22,7q36,8q13q22,10p12p15,11q13q22,14q12q24,17q21q25,20p13, 20q11q13),dim(2p23p25,3p13p21,4p13p16,5q13q21,5q33q35,6q21q22,6q25q27,8q24,9p24q34,10q11q25, 13q12q34,14q31q32,16q24,18q12q23),amp(1q25q41,7p14p21,17q22q24)	+	+	-	+	-	Luminal A
49	rev ish enh(8p11p21,20q11q13),dim(3p13p24,6q12q27,8p22p23,11q23q25,13q14q33),amp(8p12p21,20q12)	+	+	-	-	-	Luminal A
50	rev ish enh(1q21q43,3q12q29,8q11q24,20p11p13,20q11q13),dim(1p13p36,2p25,4p12p15,4q12q35,6q13q22, 7q22q36,8p12p23,15q15q22,18p11,18q11q23),amp(1q21q43,8q11q24)	+	+	+	-	-	Luminal B
51	rev ish enh(1q12q43,8p22p23),dim(4p16,9p13p24)	+	+	-	-	?	Luminal A
52	rev ish enh(1q21,1q25q41,11q13q21,14q32,15q11q14),dim(Xp11p22,Xq13q27,3p14p24,10q11q24)	+	+	-	-	-	Luminal A
53	rev ish enh(1q12q43,5p13p15,5q11q35,8q11q24,12p11p13,12q12q24,14q11q24,16p11p13,17q21q25,20p12p13, 20q11q13),dim(1p32p36,6q21q23,8p12p23,9p12p21,11q23q24,14q24q32,18p11q22,22q13),amp(1q31,8q23q24, 17q21q24)	+	+	+	-	-	Luminal B
54	rev ish enh(1p22p36,1q21q25,1q32q42,2p12,2p22p24,2q11q21,3q21,6p21p25,6q25q27,8q13q24,10p11p15, 10q11q21,10q25q26,11p11p14,11q14q23,12p13,15q22,18q12q23,19q13,20q11q13,21q22),dim(2q37,5q22q23)	-	-	-	-	+	Basal-like
55	rev ish enh(4q13q35,8p21q24,11p11p15),dim(4p13p16,6p12p24,6q13q27,8p21p23,11q14q25,17p12p13)	+	+	-	-	-	Luminal A
56	rev ish enh(1p13p21,1q21,1q25q42,6p21p25,8p11p21,10p12p15,10q22,11p11p14,11q13q22,12q24,14q24, 16p11p12,16q12,17q12q25),dim(1p22p34,1p36,4p13p16,8p22p23,11q23q25,17p12p13,18q12q21), amp(8p12p21,17q21q24)	+	-	+	-	-	Luminal B
57	rev ish enh(1q12q24,1q31q42,3q12q13,4q28q35,6p21p22,11q13,12q15q23,17q21q25,18q21q23,20q11q13), dim(1p32p36,3p13p21,3q21q25,6q25q27,11p15,11q23q24,12p12p13,13q12q22,16q11q24,17p11q12,19, 22q11q13),amp(1q21,3q12q13,17q21q24)	+	-	-	+	-	Luminal A
58	rev ish enh(1q12q42,7p12p21,7q11),dim(1p21p31,7q21q36,16q12q24),amp(7p14p15)	+	+	-	-	-	Luminal A
59	rev ish enh (1q21q43)	-	-	-	+	+	Basal-like
60	No alterations	+	+	-	-	-	Luminal A
61	rev ish enh(Xq26q28,1p13p32,1q12q42,2p12p24,2q31q37,3q12,3q26q29,4q28q31,5p13p15,5q35,6p21, 6q21q24,7p15,7q11q31,8p11p21,8q22q24,10p13p15,12q13,12q24,14q11q12,17q21q25,18p11,18q11q22, 20p11p13,20q11q13),dim(1p36,3p14p25,3q21q24,4p13p16,4q21q24,5q13q33,9p21p23,9q12q21,10q22q26, 11p12p15,11q23q25,12p13,12q21q24,13q12q34,14q24q31,15q22q24,16q11q24,17p11p13,19p13q13,21q21q22, 22q13),amp(5p15,8q23q24,17q21q24)	+	+	+	-	-	Luminal B
62	rev ish enh(17q11q12),dim(16q12q24)	+	+	+	+	-	Luminal B
63	rev ish enh(1p21p32,1q12q22,1q42q43,2p14p16,3q12q29,5p13p15,6p25q21,8p12q24,9p13p21,11p15, 11q13q23,13q21q34,17q23q25),dim(Xq23q26,1p36,3p21,4p21q13,5q13q32,6q25q27,8p22p23,13q12q13,14q24 q32,15q11q22),amp(8p12,11q14q22)	-	-	-	+	+	Basal-like
64	rev ish enh(1q12q43,3p21p26,3q12q13,3q26q27,5p13p14,6q12q27,7p15p22,8q11q24,9p12p13,9q13q34, 10p13p14,11p11,11q14,14q11q21,16p13q23,17q11q21,17q22q24,20p11p13,20q11q13),dim(Xp22,1p13p36, 3q21q24,4p12p16,5q13q31,5q33q35,6p21,8p21p23,9p22p24,10q11q26,11p12p15,11q23q25,12p11p13,12q24, 14q24q32,15q11q26,17p12p13,18p11q22,19p13)	-	-	+	+	-	ERBB2 positive
65	rev ish enh(6p12q12,8q13,8q22q24,12p12p13,12q13q24,16p11p13,17,22q11q13),dim(6q13q27,11p11p15, 11q14q25,13q12q34,14q11q32,16q12q24)	+	+	-	+	-	Luminal A

CASE	CGH FINDINGS	ER[a]	PgR[a]	ERBB2[b]	CKs 5/6[a]	CK14[a]	MOLECULAR CLASSIFICATION
66	rev ish enh(1q12q31,1q42,3p13p21,3q21q29,8p11p12,8q22q24,9p21q21,10p13p14,18q21q22),dim(4q31q34, 5q35,8p22p23)	-	-	-	+	+	Basal-like
67	rev ish enh(8p11p21,8q13q24,10p12p15),dim(8p22p23)	+	+	-	-	-	Luminal A
68	No alterations	+	-	-	+	-	Luminal A
69	rev ish enh(1q32,4q21,8q23q24,11p12p15,11q13q23,12q13q14,17q12q24),dim(Xq21q27,1p13p36,2q21q37, 9p13p24,9q12q34,11q23q25,16q12q24,22q11q13),amp(11p15,11q13q22)	+	+	-	-	-	Luminal A
70	rev ish enh(1p34,1q31q42,2p23p25,3q21q28,5p14,5q14q23,6p22p24,8q11q24,10q24q26,12q14,14q32, 17q12q24,21q21),dim(1p13p31,1p36,2q12q37,4p13p16,5q31q32,8p21p23,9p21p24,9q21q22,11q14q25,12q24, 15q15q22,17p11p13,19p13,22q11q13),amp(8q21q23)	+	+	+	-	-	Luminal B
71	rev ish enh(1q12q43,22q11q13),dim(8p21p23,8q11q13,9p13p23,9q13q34,11p12p15,13q13q34,16q11q24, 17p11p13,17q11q25,18p11,18q11q23)	+	-	-	-	-	Luminal A
72	rev ish enh(1p32p35,1q12q42,3q12q21,8q23q24,17q12q21,19q13),dim(4q31q35,8p12p22,10q25q26)	-	-	+	-	-	ERBB2 positive
73	rev ish enh(1q21q42,8, 11p11p15,11q13q14,16p12p13),dim(1p13p36,7,11q22q25,16q12q24)	+	+	-	-	-	Luminal A
74	rev ish enh(1q12q43,3q26q29,9q12q34,12p11p13,17q12q21),dim(4p15p16,10q26,11q24,14q32)	-	-	-	+	+	Basal-like
75	rev ish enh(8p11p21,16p11p13,20q11q13),dim(11q23)	+	+	-	-	-	Luminal A
76	rev ish enh(Xq13q28,2p23p24,2q12q21,3p13p24,3q12q24,4p13p16,4q21q25,4q28q31,5p13p15,7p13p21, 7q11q36,8p21q24,11q13q14,12q14,14q12q31,15q15,15q22,17q12q21,18q11q22,20p11p13,20q11q13), dim(3q26,6p11p25,6q13q27,8p21p23,10q21q26,11q22q25,12p12p13,12q21q24,15q24q26,16q12q24,17p12p13, 21q22,22q13),amp(8p11p12)	+	+	+	-	-	Luminal B
77	rev ish enh(Xq26q28,3q12q29,16p13,17q25,20q11q13),dim(1p22q31,3p21p25,8p12p23,9p22p24,11q22q24, 15q11q15,17p13,18p11q22,22q12q13)	+	-	-	+	-	Luminal A
78	rev ish enh(3q11q28,5q13q35,8q11q21,8q23q24,10p11p13,17p12,17q12q25,20q11q13),dim(3p21p26,7p21p22, 7q32q35,8p21p23,9q22q34,16q12q24)	+	+	+	-	-	Luminal B
79	rev ish enh(1q12q43),dim(16q12q24)	+	+	-	-	-	Luminal A
80	No alterations	+	+	-	-	-	Luminal A
81	rev ish enh(1q12q43,8q12q24,16p11p13,17q12q21,21q21q22),dim(17p13)	-	+	+	-	-	Luminal B
82	rev ish enh(1q22q32,1q42,8p21q24,17q21q25,19q13,20,22q13),dim(1p36,2p23,6q21,9p21p22,9q22q33, 10p11p13,10q11q21,10q23q24,11q22q25,13q12q34,14q24q31,15q11q22,18q12q22,21q21),amp(8p11p21, 8q23q24)	?	?	+	?	?	Unclassifiable
83	rev ish enh(1q21q42,4q21q24,4q32q34,13q34,15q22q26,17q21q25)	+	+	+	-	-	Luminal B
84	rev ish enh(1p13p36,1q21q43,3q12q29,9p13p24,9q21q34,16p11p13,20p11p13,20q11q13),dim(3p21p24, 4q12q35,6q12q23,7q11q36,17p11p13,17q11q12,17q23q25,22q13),amp(3q21q29)	+	+	-	-	-	Luminal A
85	rev ish enh(1p21p32,1q12q42,2p13p25,3q22q29,7p15p21,7q21q22,8q11q21,8q23q24,9p21p23,9q12q21, 9q31q34,11q13q22,16p12p13,17q21q24,18p11),dim(Xp11p22,Xq12q27,1p36,3p21,4,5p13p15,5q13q34, 8p12p23,12p12,12q12q24,14q11q24,15q21q22,16q24,17p11p13),amp(8q23)	-	-	-	-	-	Basal-like*
86	rev ish enh(2p11p12,2q11,3q11q29,4q26q31,8q11q24, 12p11p13,15q22q25,17q21q25),dim(Xp11p22, Xq21q28,3p21p24,5q12q35,8p21p23,10p11p15,10q21q26,11q13,11q23,14q12q32,15q11q21,17p13,17q12q21), amp(8q11q21,8q24,12p12p13)	-	-	-	-	+	Basal-like
87	rev ish enh(1q21q42),dim(6p12p21,6q12q21,6q25q27,16q12q24,22q12q13)	+	+	-	-	-	Luminal A

CASE	CGH FINDINGS	ER[a]	PgR[a]	ERBB2[b]	CKs 5/6[a]	CK14[a]	MOLECULAR CLASSIFICATION
88	rev ish enh(3p21p25,3q21q24,3q26q27,4p13p16,4q21q25,4q28q35,5p13p15,5q12q35,8p11p21,8q11q24, 16q12,17q11q25,20),dim(8p22p23,11p12p15,11q12q25,13q12q34,14q11q32,16q13q24)	+	+	+	-	-	Luminal B
89	rev ish enh(1q12q42,10q22q23,14q32,17q11q21),dim(8p21)	-	-	-	-	-	Basal-like*
90	rev ish enh(1q32,3p25p26,3q29,8q22q24,10p11p15,10q21,10q26,12q13q24,14q11q24,14q32,16p12p13, 17q12q25,20q13,21q22),dim(1p13,1p22p31,3p13p21,4p12p16,7q11q21,9p13p21,11p11p15,11q21q25,17p13, 18q12q22),amp(10p12p13)	+	+	+	-	-	Luminal B
91	rev ish dim(11p11p14,11q22q24,16q12q24)	+	+	-	-	-	Luminal A
92	rev ish enh(2p14p16,3q21q29,7p15p22,8q21q24,10q11q21,10q25q26,13q14q21,13q31q34,18p11,20p12), dim(8p21p22,11q23q25,18q22)	-	-	-	+	+	Basal-like
93	rev ish enh(1q12q43),dim(6q13,11q23q24,16q11q24,17q21q25)	+	+	-	-	-	Luminal A
94	rev ish enh(1q12q43,8p23q24,16p11p13,16q12q24,20p11p12,20q11q13),dim(1p36,2q21,2q31,4p16,4q31q34, 6q13q23,9q34,15q22q26,22q11q13)	+	+	-	-	-	Luminal A
95	rev ish enh(1p13p35,1q12q43,2q33q36,6q25q27,8q21q24,13q34,15q22q26,17q21q25,18q21q23, 20q12q13),dim(Xp11p22,1p36,3p13p21,6q13q21,7q21q36,8p11p21,11p15,11q21q25,13q12q14,14q24q32, 16q12q24,17p13,22q11q12),amp(8q22q24)	+	+	-	+	-	Luminal A
96	rev ish enh(3q25q29,8p11p12,8q12q24,16p13,20q11q13),dim(Xq12q21,1p22p34,1p36,3p21,4q28,7q21q36, 8p21p23,9p22p24,11q23q25,16q24,17p12p13,18q21q22)	+	+	-	-	-	Luminal A
97	rev ish enh(1q21q32,7p13p22,7q11q36,11p15,11q13),dim(1q42q43,6q24,11q23,22q12q13)	+	+	-	-	-	Luminal A
98	rev ish enh(1q21q42),dim(11q23q25,16q24)	+	+	-	-	-	Luminal A
99	rev ish enh(1q21q41,2p14p22,3q26q28,4q12q21,5p13p15,6p21p25,7p13p22,8q22q24,12q14q23,17q21q25, 18q21q22,20q12q13,21q22),dim(1p36,4q28q35,5q14q21,5q32q34,9p13p23,11p12p15,11q23q24,13q21q33, 15q22q25,16q11q24,17p13q12,21q21),amp(5p14p15,8q23q24)	+	-	-	?	-	Luminal A
100	rev ish enh(1q12q43,8p11q24),dim(8p23),amp(1q21q31)	-	-	-	+	-	Basal-like
101	rev ish enh(1q21q42,3q12q28,5q23,5q32q35),dim(13q21q33)	+	+	-	-	-	Luminal A
102	rev ish enh(1p31p33,1q42,2p12p21,3q22q29,5p13p15,7q22q36,8q12q24,9q21,17q21q24,20p13,21q21q22), dim(Xp11p21,Xq12q13,Xq23q25,Xq27,5q33q35,8p21p23,13q12q13,14q24,16q11q23),amp(3q26q27,5p14)	?	?	-	?	?	Unclassifiable
103	rev ish enh(8p12,8q11q24,11q13q22,12q14q24, 20q11q13),dim(6q13q21,6q24q27,14q24q32)	+	+	-	-	-	Luminal A
104	rev ish enh(8p11p21,8q11q24,12q12q14,16p11p13),dim(11p15)	+	+	+	-	-	Luminal B
105	rev ish enh(1q12q43,3q12q13,3q26,5q22q23,6p21p23,8p22p23,8q11q24,12q13q23,14q24,15q21,17q21q25, 20q11q13,22q11q13),dim(4q27q31,6q13q27,8p12p21,9p13p23,11q13q24,17p13q12,19p13),amp(8p23,8q21q24, 12q14q15,17q22q24)	+	-	-	-	-	Luminal A
106	rev ish enh(17q11q21)	-	-	+	-	-	ERBB2 positive
107	rev ish enh(1q12q43,2p11p25,3q13q29,5p13p15,5q12q21,5q34q35,7p13p22,8q11q24,9q21q22,13q12q34, 15q22,17q11q25,20q11q13,21q21q22),dim(Xp11p22,Xq13q26,1p13p31,1p36,4p12p16,6q13q27,8p21p23, 9p13p24,10q26,11q23q25,12p11p13,17p13,18q12q22),amp(17q12q24)	-	-	+	?	-	ERBB2 positive
108	rev ish enh(8q11q24,16p11q12),dim(8p21p23,12q24,16q24),amp(8q12q24)	+	+	-	-	-	Luminal A
109	rev ish enh(1q21,6p12p21,6q21q22,6q25q27,8p11p21,8q21q24,16p13q12,19q13,20p12q13, 21q22),dim(3p12p14,10p13,11q23,12p13,13q31q33,15q12q14,15q21q22),amp(8p21,8q21q24)	+	+	-	-	-	Luminal A

CASE	CGH FINDINGS	ER[a]	PgR[a]	ERBB2[b]	CKs 5/6[a]	CK14[a]	MOLECULAR CLASSIFICATION
110	rev ish enh(16p11p13),dim(6q22q24,16q12q24,22q11q13)	+	+	-	-	-	Luminal A
111	rev ish enh(8p11p21,12q14q24),dim(8p22p23,9p12p23,9q13q34,13q12q33,14q12q21,14q24q32,16p12, 16q11q24,17p12p13,18p11,18q11q21),amp(8p11p21)	+	+	-	-	-	Luminal A
112	rev ish enh(7p22q32,13q12,14q11q21,14q24q32),dim(13q14q34,17p12p13,17q12q25),amp(7q11,7q31,13q12)	+	+	-	-	-	Luminal A
113	rev ish enh(7q32q35,8p21q24,20q11q12,21q21q22),dim(11q23q25,18q21)	?	?	-	?	?	Unclassifiable
114	rev ish enh(1q42q43,7p14p21,12q14q15,19p13),dim(4q25q31,8p21p23,12q24,15q15q26,16q12q24,17p12p13, 22q12q13),amp(7p15)	-	-	-	-	-	Basal-like*
115	rev ish enh(16p12p13,17q21),dim(1p35p36,3p13p25,4q22q28,6q13q27,16q12q24,17p11p13)	+	+	+	?	?	Luminal B
116	rev ish enh(2p22p24,2q11,4q21,5q31q35,6p12p21,6q13,6q21q27,7p15q36,8p12p21,8q12q24,10p11p15, 10q11q22,12p12p13,12q13q24,13q12,15q15q22,16p11p13,17q21q25,19q13,20p12,20q12q13),dim(Xq21q22, 3p13p21,8p21p23,10q24q26,11p15,11q13q14,11q22q25,16q12q24,17p12p13,18q12q22,22q13),amp(6q22q24, 7p12p13,8q24,12q14q15,20q12q13)	+	-	-	+	-	Luminal A
117	rev ish enh(1q21q24,1q42q43,2p22p25,2q11q14,3q22q28,7q36,10p12p15,17q12q21,19q13),dim(4q32q34, 12q24)	-	-	+	+	+	ERBB2 positive
118	rev ish enh(3q25,8q22,10p11p15,10q21q22)	-	-	-	+	+	Basal-like
119	rev ish enh(1q21q42,8q22),dim(Xq13q21,8p21p23,11q23q25,15q11q22,18p11)	-	-	+	+	-	ERBB2 positive
120	rev ish enh(1q31q32)	+	+	-	-	-	Luminal A
121	rev ish enh(1q21q42,17q12q25)	?	?	+	?	?	Unclassifiable
122	rev ish enh(1q12q43,5p13p15,8q24,16p11p12),dim(1p21p36,2p23,4q21,6q14q21,6q23q27,9q22q33,16q12q23, 22q12)	+	+	+	-	-	Luminal B
123	rev ish enh(Xq13q27,1p12p35,1q12q43,2q22q24,3p12p13,3p21p24,3q12q27,5p14,6p25q16,7p15p21,7q31q36, 8p12q24,9p13p24,9q34,11q22q23,18q12q22,20q12q13),dim(2q37,5q13q35,8p21p23,12q24,13q14q34, 14q11q24,14q32,15q11q26,17),amp(1p22p31,1q21q42,8p11q24)	?	?	-	?	?	Unclassifiable
124	rev ish enh(1q21q43),dim(16q12q24,17p12p13)	+	+	-	-	-	Luminal A
125	rev ish enh(1q22q41,8q22q24,17q12q21),dim(Xq11,8p11p23)	-	-	+	+	-	ERBB2 positive
126	rev ish enh(1p32p35,1q21q42,5q13q33,5q35,7p13p15,7q11q32,10p11p14,10q21q23,10q26,11q13q14, 12p11p13,12q13q24,14q11q24,20p12,20q11q13),dim(Xq23q26,3p13p24,4q28q35,6p12p21,8p11p23,13q12q34, 15q11q14),amp(14q12q24)	?	?	-	?	?	Unclassifiable
127	rev ish enh(4q12q21,16q11q24,18p11)	+	+	-	-	-	Luminal A
128	rev ish enh(1q12q42,5p13p15,5q12q35,8p21,8q24,14q24q31,16p11p13,20p11p13,20q11q13),dim(11p12p14, 11q13q23)	+	+	+	-	-	Luminal B
129	rev ish enh(1q12q43,16p11p13,20p11p12,20q11q13),dim(1p13p36,3p22p24,11p11p15,11q13q25, 12p12,13q12q34,16q12q24)	+	+	-	-	-	Luminal Ale
130	rev ish enh(1q21q43,2p13p25,3p12p26,5p12p15,6p21q12,7,8q11q21,10p11p15,11q13,13q12q34,15q22q26, 17q22q25,19p13q13,20p11p13,20q11q13,21q21q22),dim(Xp11p22,Xq21q27,4p12p16,4q26q28,4q32q35, 5q12q35,8p21p23,9q31q34,10q22q26,12q13q14,14q24q31,15q11q21,18q21q23,22q13),amp(5p15,6p12p21, 7p12p13,10p11p15)	-	-	-	+	-	Basal-like
131	rev ish enh(1q121q43,7p15q22,10p11,10p13p15,10q22q23),dim(10q25,13q22q33)	+	+	-	-	-	Luminal A

CASE	CGH FINDINGS	ER[a]	PgR[a]	ERBB2[b]	CKs 5/6[a]	CK14[a]	MOLECULAR CLASSIFICATION
132	rev ish enh(7p12p22,7q11q21,7q31q36,16p11p13,20p11p13,20q11q13),dim(1p34p35,3p21p24,11q22q24, 16q12q24,17p11p13,21q11q22,22q11q12),amp(7q32)	+	+	-	?	-	Luminal A
133	rev ish enh(1q12q43,5p13p15,5q13q35,16p11p13,17q21q25,18q12q22),dim(16q11q24),amp(1q21q32)	?	?	+	?	?	Unclassifiable
134	rev ish enh(1p13p32,1q21,1q25q41,2p13p21,2q12q36,3q11q29,4p13p16,4q13q34,5p15,7p15p21, 7q31q35,8p21,8q13q24,10p11,10q11q22,10q24q26,11q14q22,12q14q23,16p11p13,20p12),dim(Xp22q21, Xq23q27,1p36,3p13p21,6,9p24q34,11q23q25,13q12q34,14q11q32,16q12q24,18,22q11q13),amp(3q12q13, 3q22q27)	+	+	-	-	-	Luminal A
135	rev ish enh(1q12q43,8q23q24,12q12q13,16p11p13,17q21q25),dim(9p21,9q12q34,18q21), amp(8q24)	+	+	+	-	-	Luminal B
136	rev ish enh(1q21q43,2p13,2p23p24,5p15,5q13q35,8p11p21,8q21q22,12p12p13,12q14q24,15q22,16p11p13, 16q12q23,17q23q25,20q11q13),dim(6p21,6q12q27, 11q23q25,13q12q14,13q31q33,14q24q31),amp(8p12p21)	+	+	+	+	+	Luminal B
137	rev ish enh(1p13p32,1q21q43,2p16q14,2q33q37,3q25q29,5p13p15,6p12p25,6q24,7p12p14,8q22q24,9p12p13, 9p22p24,11p12p14,11q13q21,13q13q14,13q22q34,16p11p13,18p11,21q21),dim(1p36,4p16,4q21q25,4q31q35, 5q13q33,8p21p23),amp(1q31,9p23p24,13q34)	?	?	-	?	-	Unclassifiable
138	rev ish enh(1q12q43,8q21q22,19q13,21q22),dim(3p14p21,4p13p16,4q31q35,6q12q27,9p12p24,10q23q26, 11q22q25,13q12q14,14q11q12,17p12p13,22q11q13)	+	-	-	+	-	Luminal A
139	rev ish enh(8q13q24,11p12p15,17q12q25),dim(13q31q34)	+	+	+	-	-	Luminal B
140	rev ish enh(20q11q13)	+	-	+	+	-	Luminal B
141	rev ish enh(11),dim(9p22p24)	+	+	-	-	-	Luminal A

Legend: [a]RE, PgR, CKs 5/6 and 14 protein expression status were evaluated by immunohistochemistry. Cases were considered positive (+) or negative (-) if ≥10% or <10% of cells were positive, respectively. [b]*ERBB2* status was evaluated by FISH or CGH. The cases scored as positive (+) presented ratios *ERRB2* gene signals to chromosome 17 centromere signals ≥2.2 on average for at least 60 tumor cells and by CGH whenever the 99% CI of the fluorescence ratio was above 1.5 in a regional gain involving 17q12 or the entire 17q arm; negative cases (-) presented ratios *ERRB2* gene signals to chromosome 17 centromere signals 1.8 - 2.2 on average for at least 100 tumor cells or <1.8 for at least 60 cells and by CGH whenever the 99% CI of the fluorescence ratio was inferior to 1.5 in 17q12. [c]Molecular classification was based on Tang *et al* (25). ?: Not analyzable. *Triple-negative cases were considered as together with basal-like group for statistical analysis.

2. OVERALL PATTERN OF GENOMIC CHANGES BY CGH

One hundred and forty one breast cancer cases were analyzed by CGH. The number of genomic imbalances ranged from 0 to 49 per case, with a median of 9.0 (Table 7).

The number of copy number gains varied from 0 to 26 (median: 5.0) and losses from 0 to 21 (median: 4.0). The chromosome arms from which material was most frequently gained were 1q (the most commonly gained bands were 1q21 and 1q32; 56.7%), 8q (45.8% at 8q24), 20q (33.1% at 20q13), 17q (29.6% at 17q21)

and 16p (28.9% at 16p12). On the other hand, the arms most often displaying loss were 16q (38.0% at 16q24), 11q (35.2% at 11q23), 8p (33.1% at 8p22), 17p (29.6% at 17p13) and 13q (20.4% at 13q31~q33). Fifty five cases (39.0%) showed amplifications of one to five discrete chromosomal regions, most frequently at 8q (18 cases), 1q and 17q (eight and seven cases, respectively), 7p and 10p (four and three cases, respectively) and 20q (two cases).

The chromosomal bands where ETS genes are located displayed the following chromosomal imbalances indicated in Table 8.

TABLE 8: Correlation of CGH imbalances at ETS gene *loci* in 141 breast carcinomas.

GENE	*LOCUS*	GAIN (%)	LOSS (%)
ETV3	1q21~q23	56.3-53.5	0
ELF3	1q32.2	56.3	0
ELK4	1q32	56.3	0
ETV5	3q28	16.2	0
ETV1	7p21.3	12	1.4
ETS1	11q23.3	3.5	35.2
FLI1	11q24.1~q24.3	2.1	31
ETV4	17q21	29.6	6.3
ETS2	21q22.2	11.3	4.9
ERG	21q22.3	11.3	4.9

3. 1q ETS COPY NUMBER GAINS BY FISH

As 1q21 and 1q32 were the two chromosome bands with most frequent genomic gains by CGH (both *loci* gained in 71 cases and additional 18 cases with copy number gain in one of the two *loci*, 9 cases each), and were the only chromosomal imbalances associated with differential expression of the ETS genes harbored in these chromosomal regions, from here our work was focused on only in the three ETS genes localized in 1q. We evaluated the copy number of the genes *ETV3* (located in 1q21~23) and *ELF3* and *ELK4* (both in 1q32) by FISH with specific BAC clones. Whereas breast carcinomas with no 1q copy number changes by CGH were also normal by FISH, this analysis showed copy number gains of the three ETS genes in 100% of the tumors with 1q imbalances that were tested (Figure 6).

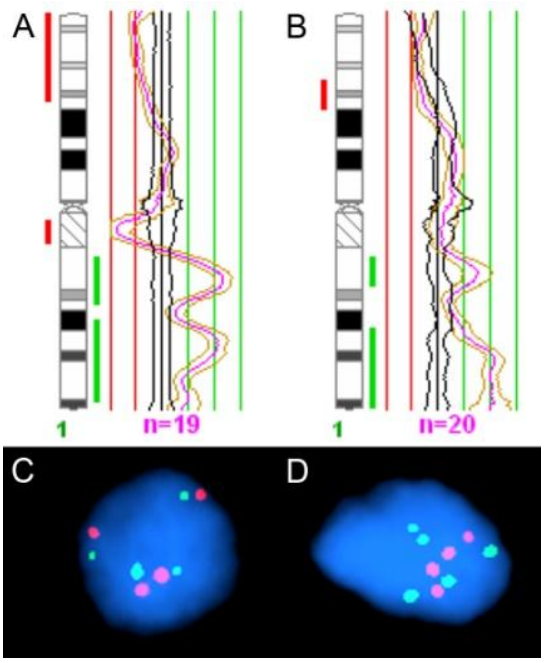

FIGURE 6: A) CGH profile of chromosome 1 showing two discrete copy number gains including 1q21~q23 (*ETV3 locus*) and 1q32 (*ELF3* and *ELK4 loci*) together with a large 1p terminal deletion. **B**) CGH profile of chromosome 1 showing two discrete copy number gains including 1q21~q23 (*ETV3 locus*) and 1q32 (*ELF3* and *ELK4 loci*) together with a small interstitial 1p deletion. **C**) Interphase FISH analysis demonstrating copy number gain of *ETV3* (green) and *ELF3* (red). **D**) Interphase FISH analysis of another breast carcinoma demonstrating copy number gain of *ETV3* (green) and *ELK4* (red).

4. ETS GENE EXPRESSION

From the 141 breast cancer cases evaluated by CGH, 114 cases were also analyzable by qRT-PCR. The expression of *ETV3* was higher in breast carcinomas with copy number gain of 1q21~q23 than in those without (Mann Whitney test; $p<0.01$), whereas the expression of *ELF3* was higher in breast carcinomas with copy number gain of 1q32 than in those without (Mann Whitney test; $p<0.01$) (Figure 7). No such association was found for *ELK4* expression and 1q32 imbalances, nor for the remaining ETS studied.

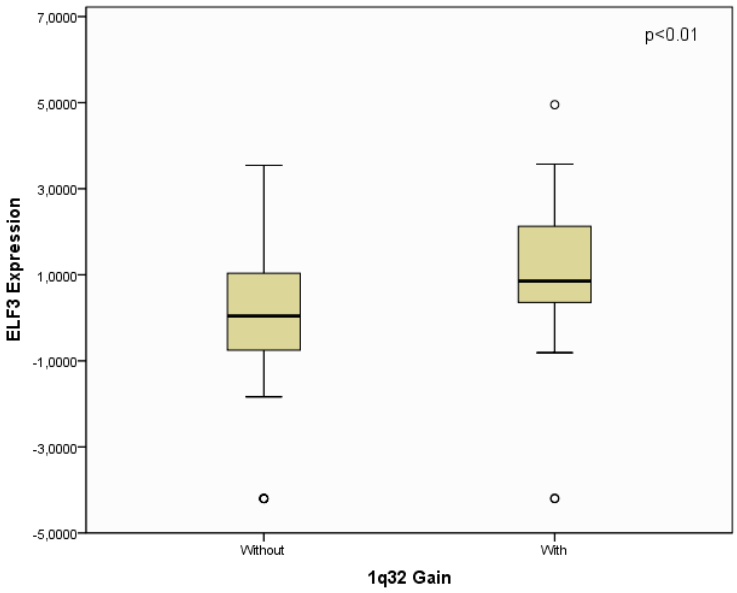

FIGURE 7: Box-plot graph showing *ELF3* expression according to the presence or absence of 1q32 copy number gain (Mann Whitney test).

5. EXPRESSION OF POTENTIAL ETS TARGET GENES

MYC expression was not associated with copy number increase at 8q24, but it was correlated with the expression of *ETV3* and *ELK4* genes (rs=0.255, $p<0.01$, for *ETV3*; rs=0.639, $p<0.01$, for *ELK4*) (Figure 8). *CRISP3* expression was not associated with copy number increase at 6p23 and it was only correlated with *ELK4* expression (rs=0.474, $p<0.01$).

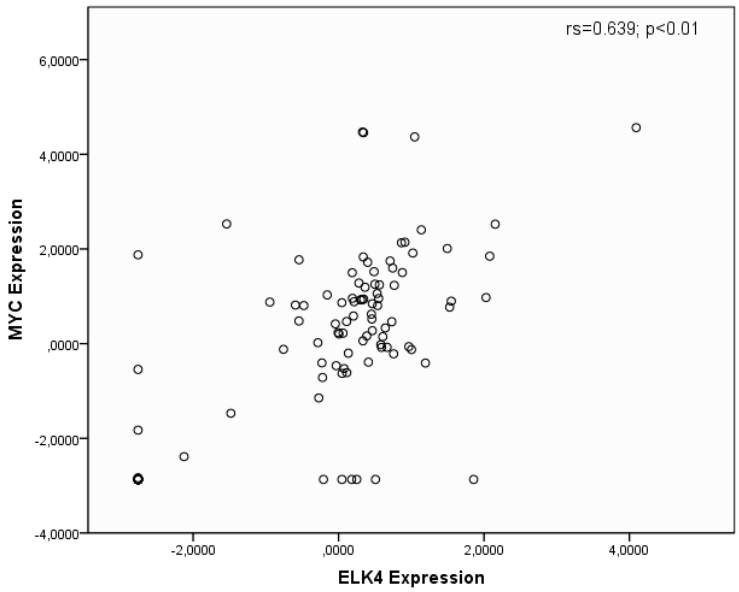

FIGURE 8: Non-parametric correlation between *ELK4* and *MYC* mRNA expression (Spearman test).

6. CLINICO-PATHOLOGICAL CORRELATIONS

Breast carcinomas were classified according to the four molecular groups defined by Perou *et al* (20) and then by Tang *et al* (25), the latter using immunohistochemical markers in parallel with cDNA microarray analyses. Breast tumor phenotype was classified in four subgroups: luminal A (ER and/or PR positive and *ERBB2* negative); luminal B (ER and/or PgR positive and *ERBB2* positive); basal-like (ER, PgR and *ERBB2* negative; and CKs 5/6 and/or 14 positive); and *ERBB2* positive (ER and PgR negative and *ERBB2* positive). Using these criteria, 71 cases were classified as luminal A (50.4%), 30 as luminal B (21.3%), 11 as *ERBB2* positive (7.8%) and 15 as basal-like (13.4%) (Table 7), which is in agreement with other published studies (26). Four cases were triple-negative and did not express CKs 5/6 or 14, but were considered together with

basal-like tumors for statistical analyses. Ten cases were unclassifiable. The basal-like molecular subtype presented the worst prognosis regarding disease-specific survival with death by disease as the end point, showing a statistically significant difference when compared with luminal B (p=0.031) and a tendency when compared with luminal A (p=0.096) (95% CI). The comparison of prognosis between the other molecular subtypes showed no significant difference, and no association between particular breast cancer molecular subtypes and the presence of 1q copy number gain or the *ETV3*, *ELF3* or *ELK4* expression levels could be observed. In the only group with a number of cases sufficient to allow the comparison (luminal A), no prognostic value was found for 1q copy number status or *ELF3* expression (Figure 9).

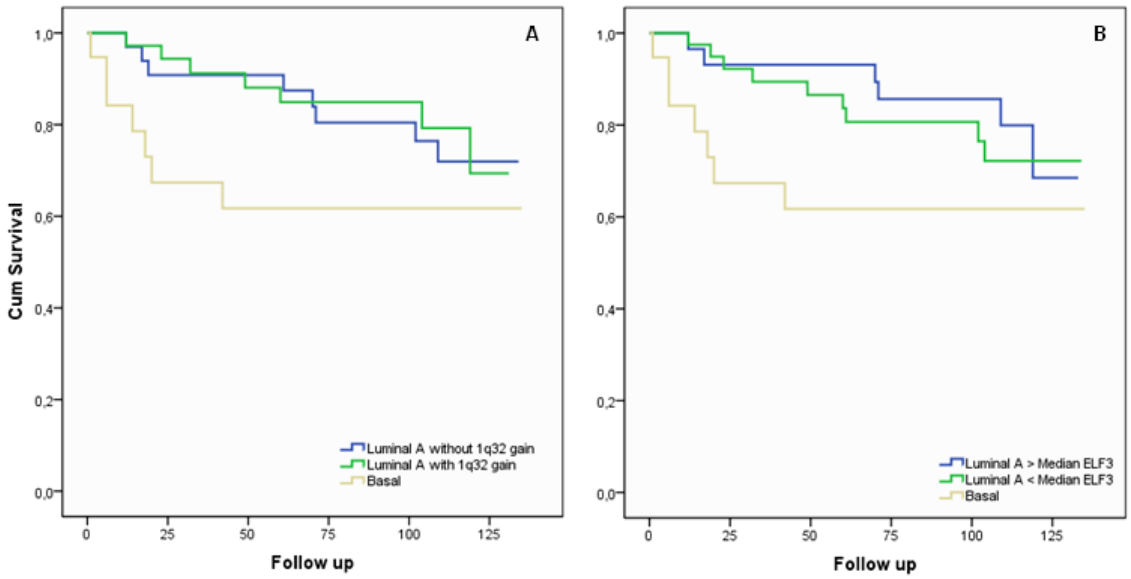

FIGURE 9: A) Comparison of disease-specific survival for 1q copy number status in luminal A breast carcinomas, as compared to the basal molecular subtype showing the worst prognosis. **B**) Comparison of disease-specific survival for *ELF3* expression above and below the mean in luminal A breast carcinomas, as compared to the basal molecular subtype showing the worst prognosis. The differences were not statistically significant.

DISCUSSION

DISCUSSION

1. 1q COPY NUMBER CHANGES AND 1q ETS EXPRESSION

Copy number gain of chromosome 1q is the most common genomic imbalance in breast carcinomas (45, 80), as confirmed by our findings in the series we here present. The target genes of the recurrent 1q gain have so far remained elusive and, given the role of the ETS family of transcription factors in several human malignancies, we asked whether the three ETS genes located in this chromosomal arm could be relevant targets in this subset of breast carcinomas. In fact, we here show that the 1q chromosome bands showing the highest frequency of copy number gains (56.7%) are exactly those harboring the three ETS *loci*, namely 1q21 (where *ETV3* is located) and 1q32 (where both *ELF3* and *ELK4* are located). Copy number gains of *ELF3*, *ETV3* and *ELK4* were subsequently confirmed by FISH with *locus* specific probes. Furthermore, we here show for the first time that there is an association between *ELF3* and *ETV3* copy number gain and overexpression of these two genes in breast carcinomas, irrespectively of the breast cancer molecular subtypes.

The same association of copy number changes and gene expression was not evident for the remaining ETS genes included in the study. In face of these results, our work focused on the 1q ETS genes and their possible role in breast cancer.

2. THE ROLE OF *ELF3* IN BREAST CARCINOGENESIS

ELF3, along with ELF5 and EHF, belongs to the clade Epithelium-Specific ETS (ESE) factor (57), a group of epithelium-restricted transcription factors with important function in epithelial cell differentiation (81, 82). *In situ* expression analysis performed in human mammary gland showed that ELF3 is expressed specifically in the epithelial cells of the ductules and lobular structures (73). Additionally, *ELF3* mRNA increased levels were detected in murine models within mammary gland epithelium during pregnancy and early lactation (73). Both evidences suggest that ELF3 may be involved in controlling processes related with cellular proliferation and differentiation, as the mammary gland undergoes extensive epithelial proliferation along with subsequent differentiation and milk

protein synthesis during pregnancy and lactation (83). This protein can also be involved in apoptosis. Studies in murines revealed that its transcript is also elevated during gland mammary involutions phases (83), which is intrinsically associated with apoptosis and remodelling pathways. Endogenous ELF3 is expressed in the cytoplasm and/or in the nucleus at very low levels, both in normal and in breast cancer tissues, as well as in benign non transformed mammary epithelial cells and in breast cell lines (73, 82, 84). Nevertheless, compiling evidence indicates that aberrant ELF3 expression may alter normal breast remodelling processes and result in tumorigenesis (73, 82, 83, 85-87).

It was previously alleged that many ETS factors exert their transforming effects in mammary epithelial cells only through nuclear transcription activity (73), but, as mentioned above, ELF3 protein is also localized within the cytoplasm in human breast cancer cells. The initiation of transformation of benign MCF-12A cells by stable expression of ELF3 is mediated by its SAR domain (serine and aspartic acid-rich domain), which acts in the cytoplasm via an unknown non-nuclear and non-transcriptional mechanism (82). On the other hand, the nuclear localization of ELF3 protein maintains the transformed mammary epithelial phenotype of fully transformed mammary epithelial cells via regulation of cellular proliferation, which is coordinately regulated by PAK-1 phosphorilation (84, 88). So, according the cellular localization, ELF3 protein has different roles in oncogenesis. Taking in account all these data, we can postulate that ELF3 initiates cellular transformation via a cytoplasmatic and PAK-1-dependent mechanism, but once fully transformed the cells require the nuclear transcription properties of ELF3 to sustain the transformed phenotype (84).

Several lines of evidence indicate that a forward autocrine regulatory loop may exist between ELF3 and growth factors, namely ERBB2, and that such a regulatory loop contributes to the regulation of cell proliferation, and ultimately to cell transformation by ELF3 (73, 84, 89-91). Regarding ELF3/ERBB2 loop, studies demonstrated that ELF3 transcription is regulated by ERBB2 receptor signaling in epithelial breast cancer cells, where expression of ERBB2 up-regulates ELF3 promoter activity, while inhibition of ERBB2 or its downstream signaling pathways decrease both ELF3 promoter activity and endogenous ELF3 protein level. It is

thus likely that the ELF3 promoter is a potential transcriptional target of the ERBB2 protein. Additionally, *ELF3* expression seems to be a downstream mediator of ERBB2 signaling and that ERBB2 induce *ELF3* gene expression in the context of breast tumorigenesis. Moreover, it has also been reported that ELF3 and ERRB2 can cooperate to confer an invasive phenotype in human mammary epithelial cells (73, 87).

Other studies described an additional role to ELF3: it was demonstrated that ELF3 can contribute to the control of TGF-β signaling pathway in human breast cancer cells, by inducing the TGF-β RII expression. Blocking ELF3 activity, the authors demonstrated a significant reduction in resistance to the growth inhibitory effects of TGF-β, which may contribute to breast carcinogenesis (92).

The seminal works performed by Gallang *et al* (67) and He *et al* (69) tried for the first time to evaluate ETS expression in breast cancer cell lines and in tumor specimens, respectively. They found increased *ELF3* mRNA levels in breast cancer cell lines and in tumor breast samples comparatively to normal breast cells. Although the paucity of breast cancer cases analyzed could bias the results (69), our data support their findings. Additionally, using FISH data, we found that the genetic mechanism promoting *ELF3* overexpression is associated with gene copy number gain/amplification.

3. THE ROLE OF *ETV3* IN BREAST CARCINOGENESIS

The role of ETV3 in breast carcinogenesis is not as well established as for ELF3. ETV3 is a member of the ETS-domain family of transcription factors. In physiological normal conditions it has been associated with the regulation of transcriptome elements of the osteoblasts (93) and in controlling the processes of proliferation and differentiation in macrophages (94). According to some evidence, the ETV3 protein is localized in the nucleus in transformed cells (95). It functions as a transcriptional repressor dependent on a C-terminal active repressor domain, blocking mitogenic responses mediated by positively acting ETS factors, such as ETS2. ETV3 binds to and represses ETS1/2 target genes, including *c-myc*, *c-myb* and *cdc-2* (96). The repressor function of ETV3 is retained even in the presence of Ras/MAPK signaling, enabling ETV3 to dominantly inhibit growth factor-dependent proliferation (96).

Other studies also demonstrated that the expression of *ETV3* in macrophages can be activated in response to RAS pathway and positively regulated by CREB-1/CREM-1, which binds to a conserved GGAA sequence found in *ETV3* (97). This core sequence represents a binding motif for members of the ETS-domain family of transcription factors, which raises the possibility that ETV3 may have the capability of transcription auto-regulation (97). In line with these results, after screening the substrates of ERK2, a MAPK protein, it was found that ETV3 was an ERK2 target in a fibroblast cell line (3T3-L1) (95). ETV3 was extensively phosphorilated by ERK2, which abrogated its capacity to bind to target genes and repress them. After searching for ETV3 binding sites throughout the genome, ETV3 target genes such as *ETV3* itself and *DDX20* were found. The ETV3 repressive antiproliferative function depends on the direct interaction with DDX20, a DEAD-box containing RNA helicase protein (94). This provides a negative feedback control mechanism, which along with constitutive instability may serve to accurately regulate ETV3 loads (95). Additionally, phosphorilation by ERK2 reduces the repression induced by ETV3, permitting the activation of cell cycle control genes, such as *MYC*, components of the NF-kB pathway and genes demanded for mRNA processing and translation (95). Once ERK2 activity ceases, neotranslated ETV3 can quickly repress its target genes, which results in a transitory rupture of transcriptional activity after ERK activation (95).

The *ETV3* mRNA transcript levels between tumor and non-tumorigenic immortalized breast cell lines were described as similar (67, 69). A probable explanation for this negative results could be the scarce number of tumor samples analyzed (69), and/or the short semi-life of ETV3 protein (about 2 hours), in conjunction with its auto-regulation capacity (95). The last hypothesis could hamper protein but also transcript detection if ETV3 in those few studied samples was not phosphorilated, and consequently was auto-repressively active. To the best of our knowledge, this is the first study that describes an association between 1q21~q23 copy number gain, both by CGH and by FISH with specific probes, and *ETV3* transcript overexpression in breast cancers. However, phosphorylation of ETV3 by ERK2, involved in MAPK signaling, abrogates its capacity to bind and repress target genes (95). It is therefore possible that overexpression of *ETV3* is an innocent bystander of 1q gain in breast carcinogenesis and that its potential

effect as a negative mitogenic regulator is contra-balanced by other oncogenic mutations that activate MAPK signaling in breast cancer.

4. THE ROLE OF *ELK4* IN BREAST CARCINOGENESIS

We also investigated the expression of the third 1q ETS gene, *ELK4*. It codifies a transcription factor that belongs to the TCF (Ternary Complex Factor) family, which in vertebrates link transcription to MAPK signaling, in partnership with serum response factors (SRF), establishing a ternary complex on the *c-Fos* promoter (98). Previous studies showed that in breast cell lines the levels of mRNA *ELK4* were stable between normal and tumor cells (67) and that *ELK4* mRNA was not detectable in breast cancer samples (69), which was justified with the non-confirmed theory of lineage-specific expression of certain ETS genes.

The expression of *ELK4* was not associated with copy number changes at its *locus*. This result is in agreement with integrative analysis of DNA copy number and mRNA expression data performed in several biological models, which show that gene copy number changes do not necessarily result in expression alterations (99-101). In prostate cancer *ELK4* overexpression may occur without an underlying genetic alteration of this gene, resulting instead from a transcriptional read-through mechanism favored by the presence of a nearby androgen responsive gene (*SLC45A3*) located upstream of *ELK4* (102, 103).

5. *MYC* AS A TARGET GENE OF 1q ETS TRANSCRIPTION FACTORS

Another goal of this work was to uncover target genes affected by altered expression of ETS transcription factors. Among the possible target genes studied, we found a correlation between *MYC* and *ETV3* and *ELK4* transcript levels.

MYC locus is 8q24.21 and as most tumors have numerous alterations in signaling cascades, MYC is likely to be deregulated by some mechanism in the majority of cancers (75). The proportion of breast cancers presenting *MYC* gene amplification (about 15%) is lower than those showing *MYC* mRNA (from 22 to 35%) or protein (approximately 45%) overexpression (37, 74). This fact can be explained by MYC being coordinated by multiple signals that regulate its promoter activity, transcriptional elongation and translation, as well as by post-transcriptional modifications (75). The fact that *MYC* expression level was not correlated with 8q

copy number gain, along with some of the evidence already described above, make us postulate that *ETV3* and *ELK4* are involved in *MYC* transcriptional regulation.

MYC is a direct ETV3 target gene. When *MYC* expression is deregulated, ETV3 exerts its suppressor effects in an attempt to directly repress it. As *MYC* mRNA levels augment, *ETV3* mRNA loads also increase in order to be translated and repress MYC. On the other hand, since *MYC* can be positively regulated by *c-Fos* (75, 104), which in turn is activated by *ELK4* (105-107), we believe that *ELK4* overexpression is relevant for breast carcinogenesis by indirectly upregulating MYC.

6. *CRISP3* AS A TARGET GENE OF 1q ETS TRANSCRIPTION FACTORS

Noteworthy is also the correlation observed between *ELK4* and *CRISP3* expression. CRISP3 belongs to the cysteine-rich secretory protein family, a highly conserved protein family among vertebrates (108). In mammals it comprises several members predominantly expressed in salivary glands and in male reproductive tract, most of which under strong androgen regulation. They were originally described in rats and it has been demonstrated that rat sperm-coating protein AEG (Crisp1 alias), which is abundantly expressed in the epididymis under strong androgen control, is involved in the rats' spermatogenesis process, post-testicular sperm maturation and capacitation event (108). Soon afterwards, screening of epididymal and salivary gland cDNA libraries allowed the isolation and characterization of CRISP1 mouse homolog and CRISP2 protein, two proteins that are strictly androgen-regulated. Additionally, *CRISP3* mRNA, which presents 77% of homology with *CRISP1*, was also identified in mouse salivary gland as being an androgen dependent transcript (108).

Human CRISP3 is a 28kDa (contains 245 aminoacids), extracellular matrix protein, detected in several tissues, with predominance in the exocrine tissues, as salivary gland, pancreas and prostate, and in less abundance in epididymis, ovary, thymus and colon (109-111). *CRISP3* gene *locus* is at 6p12.3, where the *CRISP1* and *CRISP2* genes are also localized. The CRISP3 protein is secreted in saliva, sweat, seminal plasma and is also a circulating plasma protein and it can be stored intracellularly in specific compartments and granules or in association with

membrane proteins in glycosyilated states (110, 111). Although the specific function is currently unknown, studies of sequence homology between CRISP3 and pathogenesis-associated proteins in plants, cellular localization and expression profile in thymus and neutrophils, which are rich in matrix degradation enzymes, resulted in a proposal of immune response related function (112). This feature can be associated with proteolysis and cellular matrix remodeling events, which in turn, when deregulated, may be associated with carcinogenesis.

Several studies revealed mRNA *CRISP3* overexpression in prostate cancer samples (72, 113-115), indicating a possible role in prostate cancer progression. Taking into account the results published in public databases of the transcriptome, *CRISP3* is also expressed in breast tissues but without a significant difference between normal and tumor samples.

ELK4 is a nuclear transcription factor androgen respondent ETS (116). Sixty to 85% of breast cancers express androgen receptors and even the tumors molecularly classified as hormone negative, express androgen receptor, namely some triple negative tumors (117, 118). However, the role of androgen receptors in breast cancers is still uncertain (117-119).

After performing *in silico* studies of *CRISP3* as a possible target gene for ETS factors, we did not find robust evidence indicating that *CRISP3* is an *ELK4* target gene. Taking this into consideration, we therefore postulate that the observed correlation between *ELK4* and *CRISP3* mRNA expression is mediated by the presence of androgen response elements in their promoters, resulting in a coordinated but independent expression of these two genes.

7. CLINICO-PATHOLOGICAL ASSOCIATIONS

The study did not find an association between particular breast cancer molecular subtypes and the presence of 1q copy number gain or the *ETV3* and *ELF3* expression levels. Furthermore, we could not find a prognostic value for 1q copy number status, nor for *ELF3* expression, which is probably the most important contributor to breast carcinogenesis among the ETS genes localized in 1q.

CONCLUSION

In summary, we here show for the first time that the most common genomic copy number gains in breast cancer, 1q21 and 1q32, are associated with overexpression of the ETS transcription factors *ETV3* and *ELF3* (but not *ELK4*) at these *loci* irrespective of molecular subtypes. Among the three 1q ETS genes, *ELF3* is the most likely target of the 1q copy number increase with a relevant role in breast carcinogenesis. Additionally, we show that there is a correlation between the expression of the oncogene *MYC*, irrespectively of copy number gain at its *loci* in 8q24, and the expression of both the transcriptional repressor *ETV3* and the androgen respondent *ELK4*, a relationship that is worth to explore hereafter.

FUTURE STUDIES

Testing the phenotypic impact after the induction in normal tissue/cells or repressing in cancer cells the 1q ETS genes would be an interesting approach to address their role in breast carcinogenesis. Moreover, to evaluate if target genes of *ETV3*, *ELF3* and *ELK4* genes are direct or indirect, we should perform ChIP (chromatin immunoprecipitation) and gene knock-out assays.

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APPENDIX

Frequent copy number gains at 1q21 and 1q32 are associated with overexpression of the ETS transcription factors ETV3 *and* ELF3 *in breast cancer irrespective of molecular subtypes*

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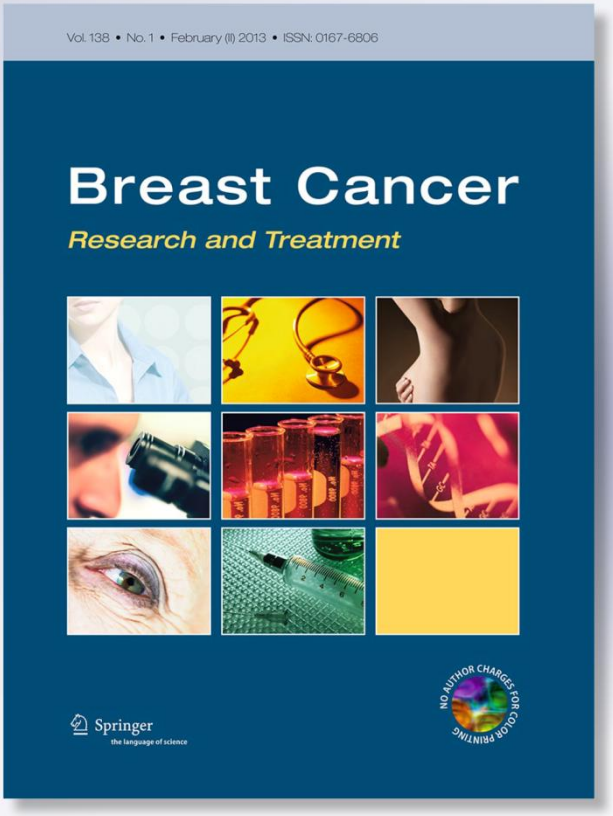

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PRECLINICAL STUDY

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Abstract Several ETS transcription factors are involved in the pathogenesis of human cancers by different mechanisms. As gene copy number gain/amplification is an alternative mechanism of oncogenic activation and 1q gain is the most common copy number change in breast carcinoma, we investigated how that genomic change impacts in the expression of the three 1q ETS family members *ETV3*, *ELK4,* and *ELF3*. We have first evaluated 141 breast carcinomas for genome-wide copy number changes by chromosomal CGH and showed that 1q21 and 1q32 were the two chromosome bands with most frequent genomic copy number gains. Second, we confirmed by FISH with locus-specific BAC clones that cases showing 1q gain/amplification by CGH showed copy number increase of the ETS genes *ETV3* (located in 1q21∼23), *ELF3,* and *ELK4* (both in 1q32). Third, gene expression levels of the three 1q ETS genes, as well as their potential targets *MYC* and *CRISP3*, were evaluated by quantitative real-time PCR. We here show for the first time that the most common genomic copy number gains in breast cancer, 1q21 and 1q32, are associated with overexpression of the ETS transcription factors *ETV3* and *ELF3* (but not *ELK4*) at these loci irrespective of molecular subtypes. Among the three 1q ETS genes, *ELF3* has a relevant role in breast carcinogenesis and is also the most likely target of the 1q copy number increase. The basal-like molecular subtype presented the worst prognosis regarding disease-specific survival, but no additional prognostic value was found for 1q copy number status or *ELF3* expression. In addition, we show that there is a correlation between the expression of the oncogene *MYC*, irrespectively of copy number gain at its loci in 8q24, and

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the expression of both the transcriptional repressor *ETV3* and the androgen respondent *ELK4*.

Keywords Breast cancer · 1q copy number gain · ETS genes · *ETV3* · *ELF3* · *ELK4*

Introduction

Several ETS (from the avian retrovirus E26—E-Twenty-Six) transcription factors are involved in the pathogenesis of human cancers by different mechanisms [1]. In sarcomas and leukemias, oncogenic activation occurs more commonly by chromosome translocations originating fusion proteins that contain the ETS DNA-binding domain, as exemplified by the hybrids *EWSR1-FLI1* and *EWSR1-ERG* associated with Ewing's sarcoma and the *TLS-ERG* and *MN1-ETV6* associated with leukemia [2, 3]. In addition, there is a broad spectrum of hematological malignancies in which the PNT oligomerization motif (of *ETV6*, for instance) is involved in chromosomal translocations with a diverse group of partners that include transcription factors and tyrosine kinases [1, 3]. More recently, ETS rearrangements have been shown to occur also in common cancers, most notably in prostate cancer, where they are found in about 50 % of the cases, most often resulting in ETS overexpression due to fusion with an androgen-dependent 5′ partner gene [4]. The most common fusion gene in prostate carcinomas is the *TMPRSS2-ERG*, but several other ETS members (namely, *ETV1*, *ETV4*, *ETV5*, and *FLI1*) have been found rearranged in this disease [4, 5].

While ETS rearrangements in breast cancer have not been identified as frequently as those in prostate cancer, the role of ETS factor expression and the expression of ETS target genes has been well-documented [6–11]. In particular, the relatively rare secretory breast carcinoma is characterized by a gene fusion involving the ETS member *ETV6* and the *NTRK3* gene, resulting from the chromosome translocation t(12;15)(p13;q25) [12]. On the other hand, breast cancer shares with prostate cancer several recurrent copy number alterations, namely losses of 8p, 13q, and 16q and gain of 8q, the latter associated with amplification of the *MYC* oncogene [13–15]. However, 1q gain is the most common copy number change in breast carcinoma, and a fraction of these show amplification [13, 14, 16]. As gene amplification is an alternative mechanism of oncogenic activation and the three ETS family members *ETV3*, *ELF3*, and *ELK4* are located in 1q, we investigated whether these ETS genes are targets of the frequent 1q copy number gains in breast cancer. Furthermore, as we and others have recently shown that the *MYC* and *CRISP3* genes are targets of ETS rearrangements in prostate cancer [5, 17, 18], we evaluated whether this association was also present for the three 1q ETS genes in breast cancer.

Materials and methods

Patients and tumor specimens

We selected the first consecutive 141 cases of a series of fresh-frozen breast carcinoma samples collected between 1999 and 2001 from patients treated at the Portuguese Oncology Institute-Porto and with follow-up data of 10 years. These samples were kept at −80 °C until molecular analyses were performed. The clinicopathological characteristics are summarized in Table 1. Paired frozen and paraffin-embedded samples were re-evaluated to assure the representativeness of cancer cells (>75 % of tumor cells). Fifteen 15 μm thick tissue sections of frozen tissue were used for DNA and RNA isolations. Sections of paraffin-embedded tissue were used as described below to analyze the estrogen (ER) and progesterone (PgR) receptors and cytokeratins 5/6 and 14 by immunohistochemistry, as well as copy number changes of *ERBB2* and of the ETS genes *ETV3*, *ELF3*, and *ELK4* by FISH.

Immunohistochemical characterization

Commercially available antibodies were used for ER (Clone 6F11; Novocastra, Newcastle, UK) and PgR (Clone 16; Novocastra, Newcastle, UK), as well as for cytokeratins 5/6 (Clones: D5 & 16B4; Cell Marque; Rocklin, CA, USA) and 14 (Clone SP53; Cell Marque; Rocklin, CA, USA), using standard methods. For each antibody appropriate positive and negative controls were used. Sections were scored as negative when <10 % and as positive when ≥10 % of stained cells were present. *ERBB2* (alias *HER2*) copy number status was determined by fluorescent in situ hybridization (FISH) and/or by comparative genomic hybridization (CGH) as described below.

Comparative genomic hybridization

DNA was extracted using standard methods and DNA quality was evaluated by agarose gel (0.8 %; w/v) electrophoresis and the quantity and purity were measured in Nanodrop (ND-1000 Spectrophotometer, Wilmington, USA). The CGH procedure described by Kallioniemi et al. [19] was performed with modifications previously described in Teixeira et al. [14] and Ribeiro et al. [20]. Copy number gains and losses were scored whenever the test and the reference 99 % CI did not overlap [21]. Amplifications were scored whenever the 99 % CI of the fluorescence ratio was above 1.5 in part of a given chromosome arm. The description of the CGH copy number changes followed the guidelines suggested by the ISCN [22].

Table 1 Clinicopathological characterization of 141 breast cancer patients

Clinicopathological characteristics	
Age at diagnosis (mean ± SE)	59.3 ± 1.2
Follow-up (mean ± SE; months)	88.9 ± 3.5
Histological Grade (Elston-Ellis)	
1	8.5 %
2	48.9 %
3	42.6 %
Histological Type	
Ductal	65.2 %
Lobular	9.2 %
Medular	1.4 %
Others	24.1 %
Pathological Stage	
I	12.1 %
IIa	36.2 %
IIb	18.4 %
IIIa	10.6 %
IIIb	9.9 %
IIIc	7.8 %
IV	5 %
Carcinoma in situ[a]	
Without	32.6 %
Not extensive	37.6 %
Extensive	28.4 %
Family history of cancer[a]	34.0 %
Family history of breast cancer[a]	13.4 %
Chemotherapy[a]	
Neoadjuvant	11.3 %
Adjuvant	56.7 %
Adjuvant radiotherapy[a]	70.9 %
Follow-up status[a]	
Alive disease free	63.8 %
Alive with disease	5.7 %
Dead disease free	21.3 %
Dead with disease	7.8 %

All patients were diagnosed before anti-ERBB2 agents became available

[a] Data not available for all patients

Fluorescent in situ hybridization

ERBB2 status was evaluated by FISH using a dual color commercial probe (Poseidon, Kreatech Diagnostics, Amsterdam, The Netherlands) in four-micron thick sections from paraffin blocks. The *ERBB2* status classification followed international consensus guidelines [23]. The cases not analyzable by FISH (15) were scored according to the CGH results, as CGH and FISH present an 82 % concordance regarding *ERBB2* status [24]. In order to evaluate copy number of ETS genes on 1q, locus-specific probes derived from bacterial artificial chromosomes (BACs) were selected according to physical and genetic mapping data on chromosomes reported in the Human Genome Browser at the University of California, Santa Cruz website (http://genome.ucsc.edu/). BACs clones targeting *ETV3* (CTD3076J23A), *ELF3* (RP11465N4B and CTD2545E14B), and *ELK4* (CTD2218H7B) were selected to cover each of the three genes and obtained from the BACPAC Resources Center (Oakland, California, USA). Adequate mapping and probe specificity of all BAC clones were confirmed by hybridization onto normal human metaphases. DNA was extracted using the Plasmid DNA Purification Kit (MACHEREY–NAGEL GmbH & Co. KG, Duren, Germany) and amplified using the GenomiPhi V2 DNA amplification kit (WGA kit, GE, Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. After a 5 min pre-treatment at 90 °C, BAC DNA was labeled with SpectrumGreen or SpectrumRed (Abbott Laboratories, IL, USA) conjugated nucleotides in nick translation reactions using the same protocol as described above for CGH. Seven hundred ng of each labeled BAC probe was then mixed with 30 µg unlabeled Cot-1 DNA (Invitrogen, Life Technologies), ethanol precipitated, dried, and dissolved in hybridization buffer (Abbott Laboratories, IL, USA). We selected cases for FISH validation according to the CGH findings, including 10 cases without 1q alterations, 10 with 1q amplifications, and 10 with 1q gain. The FISH technique was performed as described above.

Gene expression

Gene expression of the three ETSs located in 1q, as well as their potential targets *MYC* and *CRISP3*, was evaluated by quantitative real-time PCR (qRT-PCR). Total RNA was extracted from frozen breast tumor samples using Trizol® Reagent (Invitrogen, Life Technologies) and PureLink® RNA Mini Kit (Invitrogen, Life Technologies) as per the manufacturer's instructions. All RNA samples were digested with TURBO™ DNAse (Ambion, Applied Biosystems, Life Technologies, Rockville, MD), according to the manufacturer's instruction, to improve the purity of the RNA and avoid genomic DNA contamination in subsequent RT-PCR. The quality of the RNA was checked by electrophoresis on 1 % (w/v) agarose gel and the quantity and the purity ratio were evaluated in a Nanodrop 1000 (ND-1000 Spectrophotometer). Samples showing intact 28S and 18S rRNA bands were selected for the analysis. In order to increase the amount of total RNA and obtain cDNA, we used Transplex®, an Whole Transcriptome

Amplification (WTA) method (Sigma-Aldrich, Saint Louis, Missouri, USA) as previously described [25]. Quantitative PCR reactions were performed on a ABI Prism 7900 HT sequence detection system, using TaqMan® Low-Density Array (TLDA) (Applied Biosystems). The primers and probes of each assay targeting the ETS genes *ETV3*, *ELF3,* and *ELK4*, their potential target genes *MYC* and *CRISP3*, and the two normalizing housekeeping genes (*18S* and *HPRT*) (Supplementary Table 1) were preloaded and dried onto designated duplicate wells. Relative expression values were obtained by the comparative Ct method [26], normalized to each internal control gene and the average relative expression level was calculated.

Statistical analysis

The non-parametric Mann–Whitney or Kruskal–Wallis tests were used to compare RNA expression levels of *ETV3*, *ELK4*, and *ELF3* in different sample groups. To assess associations of continuous variables, the Spearman non-parametric correlation test (*rs*) was used. Kaplan Meyer curves and log rank tests were used to evaluate differences between disease survival and breast cancer molecular subtypes. Correlations between 1q gain and breast cancer molecular subtypes were evaluated using the Chi square test. A p value smaller than 0.05 was considered statistically significant. Statistical analyses were performed using the Statistical Package for Social Sciences software, version 19.0 (SPSS Inc., Chicago, IL, USA).

Results

Overall pattern of genomic changes by CGH

One hundred and forty-one breast cancer cases were analyzed by CGH. The number of genomic imbalances ranged from 0 to 49 per case, with a median of 9.0 (Supplementary Table 2). The number of copy number gains varied from 0 to 26 (median: 5.0) and losses from 0 to 21 (median: 4.0). The chromosome arms from which the material was most frequently gained were 1q (the most commonly gained bands were 1q21 and 1q32; 56.7 %), 8q (45.8 % at 8q24), 20q (33.1 % at 20q13), 17q (29.6 % at 17q21), and 16p (28.9 % at 16p12). On the other hand, the arms most often displaying loss were 16q (38.0 % at 16q24), 11q (35.2 % at 11q23), 8p (33.1 % at 8p22), 17p (29.6 % at 17p13), and 13q (20.4 % at 13q31∼q33). Fifty-five cases (39.0 %) showed amplifications of one to five discrete chromosomal regions, most frequently at 8q (18 cases), 1q, and 17q (eight and seven cases, respectively), 7p and 10p (four and three cases, respectively) and 20q (two cases) (Fig. 1a).

ERBB2 status and immunohistochemistry

Ninety-six cases were considered *ERBB2* amplification negative (68.1 %) and 45 positive (31.9 %) (Fig. 1b). Regarding the hormone receptor status, 70.2 % (99 cases) and 58.9 % (83 cases) were considered ER and PgR positive, respectively. Thirty cases (22.9 %) were hormonal receptor negative (both ER and PgR), 81 cases (61.8 %) were positive for both hormonal receptors, and 20 cases (15.2 %) were positive for one of them (18 ER positive/PgR negative; two RE negative/PgR positive). Regarding cytokeratin expression, 32 cases (25.6 %) were considered positive, 93 (66 %) were negative, and 16 were not analyzable for cytokeratin 5/6 expression, with the findings for cytokeratin 14 being 15 (10.6 %), 113 (80.1 %), and 13, respectively (Supplementary Table 2).

1q ETS copy number gains by FISH

As 1q21 and 1q32 were the two chromosome bands with most frequent genomic gains by CGH (both loci gained together in 71 cases and additional 18 cases with copy number gain in one of the two loci, 9 cases each), we evaluated the copy number of the genes *ETV3* (located in 1q21∼23) and *ELF3* and *ELK4* (both in 1q32) by FISH with specific BAC clones. Whereas breast carcinomas with no 1q copy number changes by CGH were also normal by FISH, this analysis showed copy number gains of the three ETS genes in 100 % of the tumors with 1q imbalances that were tested (Fig. 2).

1q ETS gene expression

From the 141 breast cancer cases evaluated by CGH, 114 cases were also analyzable by qRT-PCR. The expression of *ETV3* was higher in breast carcinomas with copy number gain of 1q21∼23 than in those without (Mann–Whitney test; $p < 0.01$) (Supplementary Fig. 1), whereas the expression of *ELF3* was higher in breast carcinomas with copy number gain of 1q32 than in those without (Mann–Whitney test; $p < 0.01$) (Fig. 3a). No such association was found for *ELK4* expression and 1q32 imbalances.

Expression of potential ETS targets

MYC expression was not associated with copy number increase at 8q24, but it was correlated with the expression of *ETV3* and *ELK4* genes ($rs = 0.255$, $p < 0.01$, for *ETV3*; $rs = 0.639$, $p < 0.01$, for *ELK4*) (Fig. 3b). *CRISP3* expression was not associated with copy number increase at 6p23 and it was only correlated with *ELK4* expression ($rs = 0.474$, $p < 0.01$) (Supplementary Fig. 2).

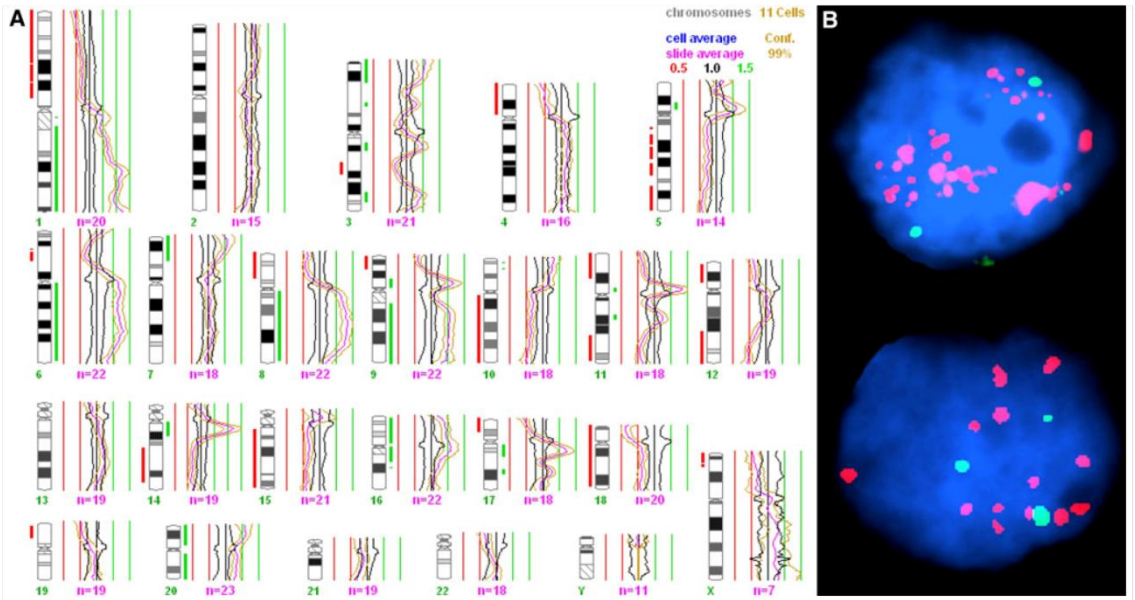

Fig. 1 **a** Comparative genomic hybridization of a breast carcinoma with several copy number changes, showing the most common chromosomal gains observed, namely 1q (where *ETV3*, *ELF3*, and *ELK4* are located), 8q (where *MYC* is located at 8q24), and 17q (where *ERBB2* is located at 17q21). Green bars to the right and red bars to the left of the chromosome ideograms indicate copy number gains and losses, respectively. **b** Interphase nuclei with *ERBB2* amplification (*red*; control in *green*)

Clinicopathological correlations

Breast carcinomas were classified according to the four molecular groups defined by Perou et al. [27] and then by Tang et al. [28] using immunohistochemical markers in parallel with cDNA microarray analyses. Breast tumor phenotype was classified in four subgroups: luminal A (ER and/or PR positive and *ERBB2* negative); luminal B (ER and/or PgR positive and *ERBB2* positive); basal-like (ER, PgR and *ERBB2* negative; and cytokeratins 5/6 and/or 14 positive); and *ERBB2* positive (ER and PgR negative and *ERBB2* positive). Using these criteria, 71 cases were classified as luminal A (50.4 %), 30 as luminal B (21.3 %), 11 as *ERBB2* positive (7.8 %) and 15 as basal-like (13.4 %), which is in agreement with other published studies [29]. Four cases were triple-negative and did not express cytokeratins 5/6 or 14, but were considered together with basal tumors for statistical analyses (Supplementary Table 2). Ten cases were unclassifiable. The basal-like molecular subtype presented the worst prognosis regarding disease-specific survival with death by disease as the end point, showing a statistically significant difference when compared with luminal B ($p = 0.031$) and a tendency when compared with luminal A ($p = 0.096$). The comparison of prognosis between the other molecular subtypes showed no significant difference, and no association between particular breast cancer molecular subtypes and the presence of 1q copy number gain or the *ETV3*, *ELF3,* or *ELK4* expression levels could be observed. In the only group with a number of cases sufficient to allow the comparison (luminal A), no prognostic value was found for 1q copy number status or ELF3 expression (Fig. 4).

Discussion

Copy number gain of chromosome 1q is the most common genomic imbalance in breast carcinomas [13, 14, 16], as confirmed by our findings in the series we here present. The target genes of the recurrent 1q gain have so far remained elusive and, given the role of the ETS family of transcription factors in several human malignancies, we asked whether the three ETS genes located in this chromosomal arm could be relevant targets in this subset of breast carcinomas. In fact, we here show that the 1q chromosome bands showing the highest frequency of copy number gains (56.7 %) are exactly those harboring the three ETS loci, namely 1q21 (where *ETV3* is located) and 1q32 (where both *ELF3* and *ELK4* are located). Copy number gains of *ELF3*, *ETV3,* and *ELK4* were subsequently confirmed by FISH with locus-specific probes. Furthermore, we here show for the first time that there is an association between *ELF3* and *ETV3* copy number gain and overexpression of

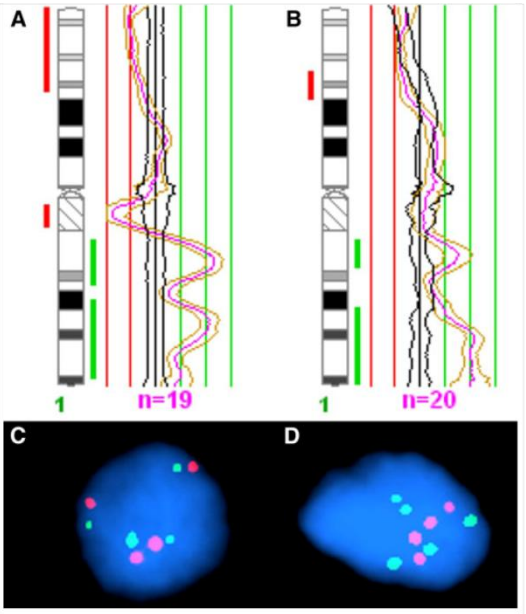

Fig. 2 **a** CGH profile of chromosome 1 showing two discrete copy number gains including 1q21∼q23 (*ETV3* locus) and 1q32 (*ELF3* and *ELK4* loci) together with a large 1p terminal deletion. **b** CGH profile of chromosome 1 showing two discrete copy number gains including 1q21∼q23 (*ETV3* locus) and 1q32 (*ELF3* and *ELK4* loci) together with a small interstitial 1p deletion. **c** Interphase FISH analysis demonstrating copy number gain of *ETV3* (*green*) and *ELF3* (*red*). **d** Interphase FISH analysis of another breast carcinoma demonstrating copy number gain of *ETV3* (*green*) and *ELK4* (*red*)

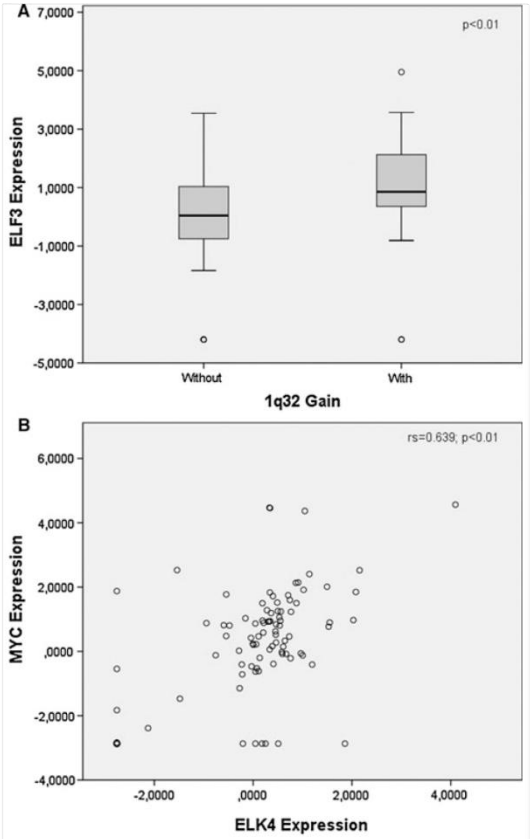

Fig. 3 **a** Box-plot graph showing *ELF3* expression according to the presence or absence of 1q32 copy number gain (Mann–Whitney test). **b** Non-parametric correlation between *ELK4* and *MYC* mRNA expression (Spearman test)

these two genes in breast carcinomas, irrespectively of the breast cancer molecular subtypes.

ELF3 is expressed specifically in the epithelial cells of the ducts and lobules in human breast tissue and *ELF3* mRNA levels are increased in mammary gland epithelium during pregnancy and early lactation in murine models [30]. These studies suggest that *ELF3* may control processes related with cellular proliferation and differentiation [31]. It has previously been shown that breast carcinomas and cell lines express more *ELF3* than normal mammary epithelial cells, although the number of samples analyzed was very small and the mechanism of overexpression was not known [32, 33]. By performing quantitative expression and copy number analyses in a large series of well-characterized primary breast carcinomas, we conclusively show that *ELF3* overexpression is closely linked with the underlying 1q32 copy number gain. In vitro evidence indicates that aberrant ELF3 expression can induce a transformed and metastatic phenotype in human breast epithelial cells [30, 31, 34–39].

The role of ETV3 in breast carcinogenesis is not as well-established as that of ELF3. Preliminary studies did not detect differences in *ETV3* mRNA transcript levels between tumor and non-tumorigenic immortalized breast cell lines in the initial screen, so its expression was not evaluated in primary breast carcinomas [32, 33]. To the best of our knowledge, this is the first study that describes an association between 1q21∼q23 gain (by CGH and FISH) and *ETV3* transcript overexpression in breast cancer. ETV3 functions as a transcriptional repressor, namely of itself, and blocks mitogenic responses by binding and repressing target genes of positively acting ETS factors, as the target gene *MYC* [40]. However, phosphorylation of ETV3 by ERK2, involved in MAPK signaling, abrogates its capacity to bind and repress target genes, thereby allowing activation of cell cycle control genes, components of the NF-kB pathway, and genes demanded for mRNA processing and translation [41].

The expression of the third 1q ETS gene we investigated was not associated with copy number changes of that

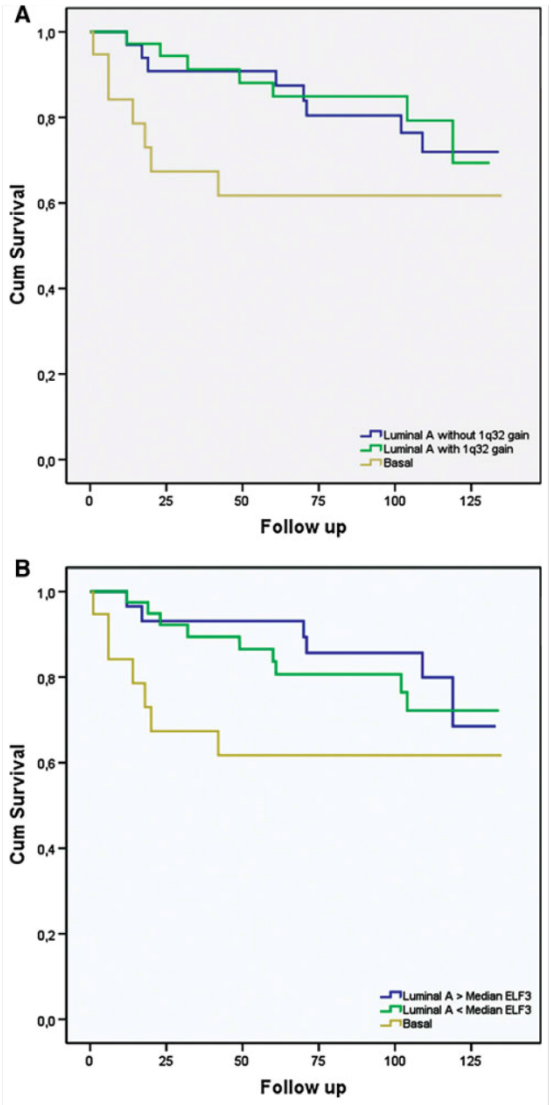

Fig. 4 **a** Comparison of disease-specific survival for 1q copy number status in luminal A breast carcinomas, as compared to the basal molecular subtype showing the worst prognosis. **b** Comparison of disease-specific survival for ELF3 expression above and below the mean in luminal A breast carcinomas, as compared to the basal molecular subtype showing the worst prognosis. The differences were not statistically significant

chromosome arm. This result is in agreement with integrative analysis of DNA copy number and mRNA expression data performed in several biological models, which show that gene copy number changes do not necessarily result in expression changes [42–44]. Earlier studies did not find major differences of *ELK4* mRNA expression between breast cancer and normal breast tissue [32, 33]. In prostate cancer *ELK4* overexpression may occur without an underlying genetic alteration of this gene, resulting instead from a transcriptional read-through mechanism favored by the presence of a nearby androgen responsive gene (*SLC45A3*) located upstream of *ELK4* [45, 46]. Interestingly, we found a correlation between *ELK4* and *CRISP3* RNA expressions, a gene belonging to the cysteine-rich secretory protein family that we and others have previously shown to be regulated by the ETS transcription factor ERG and by androgens [18, 47–49]. We found no evidence that copy number changes of 6p12, the locus of *CRISP3*, play a significant role in its expression pattern in breast cancer, and *ELK4* does not seem to regulate *CRISP3* directly. On the other hand, *ELK4* is an androgen responsive ETS transcription factor [50] and, although the role of androgen receptors in breast cancers is still uncertain [51–53], 60–85 % of breast cancers express androgen receptors [51]. We therefore postulate that the observed correlation between *ELK4* and *CRISP3* mRNA expression is mediated by the presence of androgen response elements in their promoters, resulting in a coordinated but independent expression of these two genes.

We also report here that there is a correlation between *MYC* and *ETV3* and *ELK4* transcript levels. Furthermore, we show that 8q24 copy number is not the major driver of *MYC* overexpression in breast carcinomas, therefore making it likely that *ETV3* and *ELK4* can be involved in *MYC* transcription regulation. *MYC* is regulated by multiple signals at its promoter, as well as by post-transcriptional modifications that control MYC's transcriptional targets and protein stability [54]. It is therefore possible that *ETV3* is overexpressed in a subset of breast carcinomas in part because it is located in the frequently gained 1q chromosome arm and in part as a negative feedback mechanism to regulate *MYC* overexpression, an attempt that is hampered by the post-translational changes carried out by the concurrent MAPK signaling activation, as discussed above. On the other hand, since *MYC* can be positively regulated by *c-Fos* [54, 55], which in turn is activated by *ELK4* [56–58], we are prompted to believe that *ELK4* overexpression is relevant for breast carcinogenesis by indirectly upregulating MYC.

In summary, we here show for the first time that the most common genomic copy number gains in breast cancer, 1q21 and 1q32, are associated with overexpression of the ETS transcription factors *ETV3* and *ELF3* (but not *ELK4*) at these loci irrespective of molecular subtypes. Among the three 1q ETS genes, *ELF3* is the most likely target of the 1q copy number increase with a relevant role in breast carcinogenesis. In addition, we show that there is a correlation between the expression of the oncogene *MYC*, irrespectively of copy number gain at its loci in 8q24, and the expression of both the transcriptional repressor *ETV3*

and the androgen respondent *ELK4*, a relationship that is worth to explore in future studies.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards The study was approved by the institutional review board.

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